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(54) Title: VACCINE COMPRISING GP120 AND NEF AND/OR TAT FOR THE IMMUNISATION AGAINST HIV

(57) Abstract: Use of a) an HIV Tat protein or polynucleotide; or b) an HIV Nef protein or polynucleotide; or c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide; and an HIV gp 120 protein or polynucleotide in the manufacture of a vaccine suitable for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans against HIV, wherein the protein or polynucleotide is delivered via a bombardment approach.

5   VACCINE COMPRISING GP120 AND NEF AND/OR TAT FOR THE IMMUNISATION AGAINST HIV

DESCRIPTION

The present invention relates to novel uses of HIV proteins in medicine and vaccine
10 compositions containing such HIV proteins. In particular, the invention relates to the
use of HIV Tat and HIV gp120 proteins in combination. Furthermore, the invention
relates to the use of HIV Nef and HIV gp120 proteins in combination. The invention
also relates to DNA encoding HIV Tat and/or Nef (hereinafter Tat DNA and/or Nef
DNA) and DNA encoding HIV gp120 (hereinafter gp120 DNA) and vectors
15 comprising such DNAs. The invention relates in particular to administering the
proteins and/or DNAs in a prime-boost schedule via a particle bombardment
approach.

HIV-1 is the primary cause of the acquired immune deficiency syndrome (AIDS)
20 which is regarded as one of the world's major health problems. Although extensive
research throughout the world has been conducted to produce a vaccine, such efforts
thus far have not been successful.

The HIV envelope glycoprotein gp120 is the viral protein that is used for attachment
25 to the host cell. This attachment is mediated by the binding to two surface molecules
of helper T cells and macrophages, known as CD4 and one of the two chemokine
receptors CCR-4 or CXCR-5. The gp120 protein is first expressed as a larger
precursor molecule (gp160), which is then cleaved post-translationally to yield gp120
and gp41. The gp120 protein is retained on the surface of the virion by linkage to the
30 gp41 molecule, which is inserted into the viral membrane.

The gp120 protein is the principal target of neutralizing antibodies, but unfortunately
the most immunogenic regions of the proteins (V3 loop) are also the most variable
parts of the protein. Therefore, the use of gp120 (or its precursor gp160) as a vaccine
35 antigen to elicit neutralizing antibodies is thought to be of limited use for a broadly
protective vaccine. The gp120 protein does also contain epitopes that are recognized
by cytotoxic T lymphocytes (CTL). These effector cells are able to eliminate virus-

5   infected cells, and therefore constitute a second major antiviral immune mechanism. In contrast to the target regions of neutralizing antibodies some CTL epitopes appear to be relatively conserved among different HIV strains. For this reason gp120 and gp160 are considered to be useful antigenic components in vaccines that aim at eliciting cell-mediated immune responses (particularly CTL).

10

Non-envelope proteins of HIV-1 have been described and include for example internal structural proteins such as the products of the *gag* and *pol* genes and, other non-structural proteins such as Rev, Nef, Vif and Tat (Greene et al., New England J. Med, **324**, 5, 308 et seq (1991) and Bryant et al. (Ed. Pizzo), Pediatr. Infect. Dis. J., **11**, 5,

15   390 et seq (1992).

HIV Tat and Nef proteins are early proteins, that is, they are expressed early in infection and in the absence of structural protein.

20   In a conference presentation (C. David Pauza, Immunization with Tat toxoid attenuates SHIV89.6PD infection in rhesus macaques, 12th Cent Gardes meeting, Marnes-La-Coquette, 26.10.1999), experiments were described in which rhesus macaques were immunised with Tat toxoid alone or in combination with an envelope glycoprotein gp160 vaccine combination (one dose recombinant vaccinia virus and

25   one dose recombinant protein). However, the results observed showed that the presence of the envelope glycoprotein gave no advantage over experiments performed with Tat alone.

However, we have found that a Tat- and/or Nef-containing immunogen (especially a

30   Nef-Tat fusion protein) acts synergistically with gp120 in protecting rhesus monkeys from a pathogenic challenge with chimeric human-simian immunodeficiency virus (SHIV). To date the SHIV infection of rhesus macaques is considered to be the most relevant animal model for human AIDS. Therefore, we have used this preclinical model to evaluate the protective efficacy of vaccines containing a gp120 antigen and a

35   Nef- and Tat-containing antigen either alone or in combination. Analysis of two markers of viral infection and pathogenicity, the percentage of CD4-positive cells in the peripheral blood and the concentration of free SHIV RNA genomes in the plasma

5   of the monkeys, indicated that the two antigens acted in synergy. Immunization with
       either gp120 or NefTat + SIV Nef alone did not result in any difference compared to
       immunization with an adjuvant alone. In contrast, the administration of the
       combination of gp120 and NefTat + SIV Nef, antigens resulted in a marked
       improvement of the two above-mentioned parameters in all animals of those particular
10   experimental group.

       As described above, the NefTat protein, the SIV Nef protein and gp120 protein
       together give an enhanced response over that which is observed when either NefTat +
       SIV Nef, or gp120 are used alone. This enhanced response, or synergy can be seen in
15   a decrease in viral load as a result of vaccination with these combined proteins.
       Alternatively, or additionally the enhanced response manifests itself by a maintenance
       of CD4+ levels over those levels found in the absence of vaccination with HIV
       NefTat, SIV Nef and HIV gp120. The synergistic effect is attributed to the
       combination of gp120 and Tat, or gp120 and Nef, or gp120 and both Nef and Tat.
20

       It has been found that not only the Nef, Tat or NefTat proteins are advantageously
       combined or administered with the gp 120 protein. The same advantages are seen
       when DNA encoding Nef, Tat or NefTat is administered with gp 120 (protein or
       corresponding DNA).
25

       It has been found that the proteins above, or DNA encoding the proteins, may
       advantageously be administered via a prime-boost strategy. In one aspect the present
       invention relates to such administration via a bombardment approach. Accordingly
       the invention provides the use of
30   a) an HIV Tat protein or polynucleotide; or
       b) an HIV Nef protein or polynucleotide; or
       c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or
       polynucleotide;
       and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine suitable
35   for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans
       against HIV, wherein the protein or polynucleotide is delivered via a bombardment
       approach.

5

Numerous methods of carrying out a particle bombardment approach are known. See for example WO 91/07487. In one illustrative example, gas-driven particle acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI),

10   some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, coated with a substance such as polynucleotide, is accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest,

15   typically the skin. The particles are preferably gold beads of a 0.4 – 4.0 μm, more preferably 0.6 – 2.0 μm diameter and the polynucleotide, preferably DNA is coated onto these and then encased in a cartridge for placing into the "gene gun".

The addition of other HIV proteins or DNA encoding them may further enhance the

20   synergistic effect, which was observed between gp120 and Tat and/or Nef. These other proteins may also act synergistically with individual components of the gp120, Tat and/or Nef-containing vaccine, not requiring the presence of the full original antigen combination. The additional proteins may be regulatory proteins of HIV such as Rev, Vif, Vpu, and Vpr. They may also be structural proteins derived from the HIV

25   *gag* or *pol* genes.

The HIV *gag* gene encodes a precursor protein p55, which can assemble spontaneously into immature virus-like particles (VLPs). The precursor is then proteolytically cleaved into the major structural proteins p24 (capsid) and p18

30   (matrix), and into several smaller proteins. Both the precursor protein p55 and its major derivatives p24 and p18 may be considered as appropriate vaccine antigens which may further enhance the synergistic effect observed between gp120 and Tat and/or Nef. The precursor p55 and the capsid protein p24 may be used as VLPs or as monomeric proteins.

35

The HIV Tat protein for use in the present invention may, optionally, be linked to an HIV Nef protein, for example as a fusion protein.

5

The HIV Tat protein, the HIV Nef protein or the NefTat fusion protein for use in the present invention may have a C terminal Histidine tail which preferably comprises between 5-10 Histidine residues. The presence of an histidine (or 'His') tail aids purification.

10

In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 10 and preferably six Histidine residues. These are advantageous in aiding purification. Separate expression, in yeast (Saccharomyces cerevisiae), of Nef (Macreadie I.G. et al., 1993, Yeast 9 (6) 565-573) and Tat (Braddock M et al., 1989,

15   Cell 58 (2) 269-79) has been reported. Nef protein and the Gag proteins p55 and p18 are myristoylated. The expression of Nef and Tat separately in a Pichia expression system (Nef-His and Tat-His constructs), and the expression of a fusion construct Nef-Tat-His have been described previously in WO99/16884.

20   The DNA and amino acid sequences of representative Nef-His (Seq. ID. No.s 8 and 9), Tat-His (Seq. ID. No.s 10 and 11) and of Nef-Tat-His fusion proteins (Seq. ID. No.s 12 and 13) are set forth in Figure 1.

The HIV proteins may be used in their native conformation, or more preferably, may

25   be modified for vaccine use. These modifications may either be required for technical reasons relating to the method of purification, or they may be used to biologically inactivate one or several functional properties of the Tat or Nef protein. Thus the invention encompasses the use of derivatives of HIV proteins or polynucleotides, particularly DNAs, which may be, for example, mutated. The term 'mutated' is used

30   herein to mean a DNA or protein molecule which has undergone deletion, addition or substitution of one or more nucleotides or amino acids using well known techniques for site directed mutagenesis or any other conventional method.

For example, a mutant Tat protein may be mutated so that it is biologically inactive

35   whilst still maintaining its immunogenic epitopes. One possible mutated tat gene, constructed by D.Clements (Tulane University), (originating from BH10 molecular

clone) bears mutations in the active site region (Lys41→Ala)and in RGD motif

(Arg78→Lys and Asp80→Glu) (Virology 235: 48-64, 1997).

A mutated Tat is illustrated in Figure 1 (Seq. ID. No.s 22 and 23) as is a Nef-Tat

Mutant-His (Seq. ID. No.s 24 and 25).

The HIV Tat or Nef proteins for use in the present invention may be modified by

chemical methods during the purification process to render the proteins stable and

monomeric. One method to prevent oxidative aggregation of a protein such as Tat or

Nef is the use of chemical modifications of the protein's thiol groups. In a first step

the disulphide bridges are reduced by treatment with a reducing agent such as DTT,

beta-mercaptoethanol, or gluthatione. In a second step the resulting thiols are blocked

by reaction with an alkylating agent (for example, the protein can be caused to react

with iodoacetamide). Such chemical modification does not modify functional

properties of Tat or Nef as assessed by cell binding assays and inhibition of

lymphoproliferation of human peripheral blood mononuclear cells.

It will be understood that the invention also emcompasses the use of fragments of the

full length proteins provided that the fragments comprise at least one immunogenic

epitope.

The HIV Tat protein and HIV gp120 proteins can be purified by the methods outlined

in the attached examples.

An immunoprotective or immunotherapeutic quantity of the Tat and/or Nef or NefTat

and gp120 components (protein or DNA) for use in the invention may be prepared by

conventional techniques.

Vaccine preparation is generally described in New Trends and Developments in

Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A.

1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S.

Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for

5   example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent
       4,474,757.

       The amount of protein in a vaccine dose is selected as an amount which induces an
       immunoprotective response without significant, adverse side effects in typical

10   vaccinees. Such amount will vary depending upon which specific immunogen is
       employed. Generally, it is expected that each dose will comprise 1-1000 μg of each
       protein, preferably 2-200 μg, most preferably 4-40 μg of Tat or Nef or NefTat and
       preferably 1-150 μg, most preferably 2-25 μg of gp120. An optimal amount for a
       particular vaccine can be ascertained by standard studies involving observation of

15   antibody titres and other responses in subjects. One particular example of a vaccine
       dose will comprise 20 μg of NefTat and 5 or 20 μg of gp120. Following an initial
       vaccination, subjects may receive a boost in about 4 weeks, and a subsequent second
       booster immunisation.

20   The proteins of the present invention are preferably adjuvanted in a vaccine
       formulation of the invention. The polynucleotides used in the present invention are
       optionally adjuvanted, and may be delivered in a formulation with an adjuvant or
       separately from the adjuvant, either simultaneously or sequentially. Adjuvants are
       described in general in Vaccine Design – the Subunit and Adjuvant Approach, edited

25   by Powell and Newman, Plenum Press, New York, 1995.

       Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum)
       or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an
       insoluble suspension of acylated tyrosine, or acylated sugars, cationically or

30   anionically derivatised polysaccharides, or polyphosphazenes.

       In the formulation of vaccines for use in the invention it is preferred that the adjuvant
       composition induces a preferential Th1 response. However it will be understood that
       other responses, including other humoral responses, are not excluded.

35

       An immune response is generated to an antigen through the interaction of the antigen
       with the cells of the immune system. The resultant immune response may be broadly

5   distinguished into two extreme catagories, being humoral or cell mediated immune
     responses (traditionally characterised by antibody and cellular effector mechanisms of
     protection respectively). These categories of response have been termed Th1-type
     responses (cell-mediated response), and Th2-type immune responses (humoral
     response).

10

     Extreme Th1-type immune responses may be characterised by the generation of
     antigen specific, haplotype restricted cytotoxic T lymphocytes, and natural killer cell
     responses. In mice Th1-type responses are often characterised by the generation of
     antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type

15   antibodies. Th2-type immune responses are characterised by the generation of a broad
     range of immunoglobulin isotypes including in mice IgG1, IgA, and IgM.

     It can be considered that the driving force behind the development of these two types
     of immune responses are cytokines, a number of identified protein messengers which

20   serve to help the cells of the immune system and steer the eventual immune response
     to either a Th1 or Th2 response. Thus high levels of Th1-type cytokines tend to
     favour the induction of cell mediated immune responses to the given antigen, whilst
     high levels of Th2-type cytokines tend to favour the induction of humoral immune
     responses to the antigen.

25

     It is important to remember that the distinction of Th1 and Th2-type immune
     responses is not absolute. In reality an individual will support an immune response
     which is described as being predominantly Th1 or predominantly Th2. However, it is
     often convenient to consider the families of cytokines in terms of that described in

30   murine CD4 +ve T cell clones by Mosmann and Coffman (*Mosmann, T.R. and
     Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion
     lead to different functional properties. Annual Review of Immunology, 7, p145-173*).
     Traditionally, Th1-type responses are associated with the production of the INF-γ and
     IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the

35   induction of Th1-type immune responses are not produced by T-cells, such as IL-12.
     In contrast, Th2- type responses are associated with the secretion of IL-4, IL-5, IL-6,
     IL-10 and tumour necrosis factor-β(TNF-β).

5   It is known that certain vaccine adjuvants are particularly suited to the stimulation of
         either Th1 or Th2 - type cytokine responses. Traditionally the best indicators of the
         Th1:Th2 balance of the immune response after a vaccination or infection includes
         direct measurement of the production of Th1 or Th2 cytokines by T lymphocytes *in*
10   *vitro* after restimulation with antigen, and/or the measurement of the IgG1:IgG2a ratio
         of antigen specific antibody responses.

         Thus, a Th1-type adjuvant is one which stimulates isolated T-cell populations to
         produce high levels of Th1-type cytokines when re-stimulated with antigen *in vitro*,
15   and induces antigen specific immunoglobulin responses associated with Th1-type
         isotype.

         Preferred Th1-type immunostimulants which may be formulated to produce adjuvants
         suitable for use in the present invention include and are not restricted to the following.
20
         Monophosphoryl lipid A, in particular 3-de-O-acylated monophosphoryl lipid A (3D-
         MPL), is a preferred Th1-type immunostimulant for use in the invention.   3D-MPL is
         a well known adjuvant manufactured by Ribi Immunochem, Montana.   Chemically it
         is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid A with either
25   4, 5, or 6 acylated chains. It can be purified and prepared by the methods taught in GB
         2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and
         3-O-deacylated variants thereof. Other purified and synthetic lipopolysaccharides have
         been described (US 6,005,099 and EP 0 729 473 B1; Hilgers *et al.*, 1986,
         Int.Arch.Allergy.Immunol., 79(4):392-6; Hilgers *et al.*, 1987, Immunology, 60(1):141-
30   6; and EP 0 549 074 B1).   A preferred form of 3D-MPL is in the form of a particulate
         formulation having a small particle size less than 0.2μm in diameter, and its method of
         manufacture is disclosed in EP 0 689 454.

         Saponins are also preferred Th1 immunostimulants in accordance with the invention.
35   Saponins are well known adjuvants and are taught in: Lacaille-Dubois, M and Wagner
         H. (1996. A review of the biological and pharmacological activities of saponins.
         Phytomedicine vol 2 pp 363-386). For example, Quil A (derived from the bark of the

5   South American tree Quillaja Saponaria Molina), and fractions thereof, are described
       in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther
       Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. The haemolytic
       saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as
       potent systemic adjuvants, and the method of their production is disclosed in US

10   Patent No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the
       use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for
       systemic vaccines. Use of QS21 is further described in Kensil *et al.* (1991. J.
       Immunology vol 146, 431-437). Combinations of QS21 and polysorbate or
       cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems

15   comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739
       and WO 96/11711.

       Another preferred immunostimulant is an immunostimulatory oligonucleotide
       containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for

20   cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as
       being an adjuvant when administered by both systemic and mucosal routes (WO
       96/02555, EP 468520, Davis *et al., J.Immunol*, 1998, 160(2):870-876; McCluskie and
       Davis, *J.Immunol.*, 1998, 161(9):4463-6). Historically, it was observed that the DNA
       fraction of BCG could exert an anti-tumour effect. In further studies, synthetic

25   oligonucleotides derived from BCG gene sequences were shown to be capable of
       inducing immunostimulatory effects (both in vitro and in vivo). The authors of these
       studies concluded that certain palindromic sequences, including a central CG motif,
       carried this activity. The central role of the CG motif in immunostimulation was later
       elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has

30   shown that the CG motif has to be in a certain sequence context, and that such
       sequences are common in bacterial DNA but are rare in vertebrate DNA. The
       immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine;
       wherein the CG motif is not methylated, but other unmethylated CpG sequences are
       known to be immunostimulatory and may be used in the present invention.

35

       In certain combinations of the six nucleotides a palindromic sequence is present.
       Several of these motifs, either as repeats of one motif or a combination of different

5   motifs, can be present in the same oligonucleotide. The presence of one or more of
these immunostimulatory sequences containing oligonucleotides can activate various
immune subsets, including natural killer cells (which produce interferon γ and have
cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Other
unmethylated CpG containing sequences not having this consensus sequence have
10   also now been shown to be immunomodulatory.

CpG when formulated into vaccines, is generally administered in free solution
together with free antigen (WO 96/02555; McCluskie and Davis, *supra*) or covalently
conjugated to an antigen (WO 98/16247), or formulated with a carrier such as
15   aluminium hydroxide ((Hepatitis surface antigen) Davis *et al. supra* ; Brazolot-Millan
et al., Proc.Natl.Acad.Sci., USA, 1998, 95(26), 15553-8).

Such immunostimulants as described above may be formulated together with carriers,
such as for example liposomes, oil in water emulsions, and or metallic salts, including
20   aluminium salts (such as aluminium hydroxide). For example, 3D-MPL may be
formulated with aluminium hydroxide (EP 0 689 454) or oil in water emulsions (WO
95/17210); QS21 may be advantageously formulated with cholesterol containing
liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO
98/15287); CpG may be formulated with alum (Davis *et al. supra* ; Brazolot-Millan
25   *supra*) or with other cationic carriers.

Combinations of immunostimulants are also preferred, in particular a combination of
a monophosphoryl lipid A and a saponin derivative (WO 94/00153; WO 95/17210;
WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the
30   combination of QS21 and 3D-MPL as disclosed in WO 94/00153. Alternatively, a
combination of CpG plus a saponin such as QS21 also forms a potent adjuvant for use
in the present invention.

Thus, suitable adjuvant systems include, for example, a combination of
35   monophosphoryl lipid A, preferably 3D-MPL, together with an aluminium salt.
An enhanced system involves the combination of a monophosphoryl lipid A and a
saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in

5   WO 94/00153, or a less reactogenic composition where the QS21 is quenched in

        cholesterol containing liposomes (DQ) as disclosed in WO 96/33739.

        A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in

        an oil in water emulsion is described in WO 95/17210 and is another preferred

10   formulation for use in the invention.

        Another preferred formulation comprises a CpG oligonucleotide alone or together

        with an aluminium salt.

15   Particularly preferred adjuvant and/or carrier combinations are as follows:
        i)   3D-MPL + QS21 in DQ
        ii)   Alum + 3D-MPL
        iii)   Alum + QS21 in DQ + 3D-MPL
        iv)   Alum + CpG
20   v)   3D-MPL + QS21 in DQ + oil in water emulsion
        vi)   CpG

        As already noted, the vaccine may contain polynucleotide, preferably DNA, encoding

        one or more of the Tat, Nef and gp120 polypeptides, such that the polypeptide is

25   generated *in situ*.

        The DNA constructs *per se*, especially those described herein, also form part of the

        invention.

30   The polynucleotide may be present within any of a variety of delivery systems known

        to those of ordinary skill in the art, including nucleic acid expression systems such as

        plasmid DNA, bacteria and viral expression systems. Numerous gene delivery

        techniques are well known in the art, such as those described by Rolland, Crit. Rev.

        Therap. Drug Carrier Systems 15:143-198, 1998 and references cited therein,

35   incorporated herein by reference.

5   Plasmid based delivery of genes, particularly for immunisation or gene therapy

purposes is known. For example, administration of naked DNA by injection into

mouse muscle is outlined by Vical in International Patent Application WO90/11092.

Johnston et al WO 91/07487 describe methods of transferring a gene to vertebrate

10   cells, by the use of microprojectiles that have been coated with a polynucleotide

encoding a gene of interest, and accelerating the microparticles such that the

microparticles can penetrate the target cell.

DNA vaccines usually consist of a bacterial plasmid vector into which is inserted a

15   strong viral promoter, the gene of interest which encodes for an antigenic peptide and

a polyadenylation/transcriptional termination sequences. The gene of interest may

encode a full protein, a fusion protein comprising different antigens, or simply an

antigenic peptide sequence relating to the pathogen, tumour or other agent which is

intended to be protected against. Thus the plasmid may encode a fragment of a full

20   protein, provided that the fragment comprises at least one immunogenic epitope of the

full protein. The plasmid can be grown in bacteria, such as for example E.coli and

then isolated and prepared in an appropriate medium, depending upon the intended

route of administration, before being administered to the host. Following

administration the plasmid is taken up by cells of the host where the encoded peptide

25   is produced. The plasmid vector will preferably be made without an origin of

replication which is functional in eukaryotic cells, in order to prevent plasmid

replication in the mammalian host and integration within chromosomal DNA of the

animal concerned. All of these features may apply singly or in combination to the

present invention.

30

There are a number of advantages of DNA vaccination relative to traditional

vaccination techniques. First, it is predicted that because the proteins which are

encoded by the DNA sequence are synthesised in the host, the structure or

conformation of the protein will be similar to the native protein associated with the

35   disease state. It is also likely that DNA vaccination will offer protection against

different strains of a virus, by generating a cytotoxic T lymphocyte response that

recognises epitopes from conserved proteins. Furthermore, because the plasmids are

5   taken up by the host cells where antigenic protein can be produced, a long-lasting immune response will be elicited. The technology also offers the possibility of combining diverse immunogens into a single preparation to facilitate simultaneous immunisation in relation to a number of disease states.

10   Helpful background information in relation to DNA vaccination is provided in Donnelly et al "DNA vaccines" Ann. Rev Immunol. 1997 15: 617-648, the disclosure of which is included herein in its entirety by way of reference.

In one preferred embodiment the DNA can be delivered via a particle bombardment

15   approach e.g. a "gene gun" approach as described hereinabove.

Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). When the expression system is a recombinant live microorganism, such as a virus or

20   bacterium, the gene of interest can be inserted into the genome of a live recombinant virus or bacterium. Inoculation and *in vivo* infection with this live vector will lead to *in vivo* expression of the antigen and induction of immune responses. Viruses and bacteria used for this purpose are for instance: poxviruses (e.g; vaccinia, fowlpox, canarypox, modified poxviruses e.g. Modified Virus Ankara (MVA)), alphaviruses

25   (Sindbis virus, Semliki Forest Virus, Venezuelian Equine Encephalitis Virus), flaviviruses (yellow fever virus, Dengue virus, Japanese encephalitis virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), herpesviruses (varicella zoster virus, etc), Listeria, Salmonella , Shigella, Neisseria, BCG. These viruses and bacteria can be virulent, or attenuated in various ways in

30   order to obtain live vaccines.

Thus, the Nef, Tat and gp120 components of a preferred vaccine according to the invention may be provided in the form of polynucleotides or recombinant DNA encoding the desired proteins. The polynucleotides employed in the invention may

35   encode a full protein, a fusion protein comprising different antigens, or one or more antigenic peptide sequences. Thus the polynucleotides may encode a fragment of a

5   full protein, provided that the fragment comprises at least one immunogenic epitope of
       the full protein.

       At least one of the DNAs for Nef, Tat, NefTat or gp 120 may preferably be codon
       optimised as described, for example, in Andre S. Seed B. Eberle J. Schraut W.
10   Bultmann A. Haas J. (1998): Increased immune response elicited by DNA
       vaccination with a synthetic gp120 sequence with optimized codon usage, Journal of
       Virology. 72(2):1497-503. In one preferred aspect the DNA encoding gp120 is codon
       optimised.

15   Codon optimisation is used to optimise the polynucleotide sequences for expression in
       mammalian cells. That is the sequence is optimised to resemble the codon usage of
       genes in mammalian cells.

       In one embodiment of the present invention the Nef, Tat, NefTat or gp120
20   polynucleotide sequence has a codon usage pattern which resembles that of highly
       expressed mammalian genes, particularly human genes. Preferably the polynucleotide
       sequence is a DNA sequence. Desirably the codon usage pattern of the polynucleotide
       sequence is typical of highly expressed human genes.

25   The DNA code has 4 letters (A, T, C and G) and uses these to spell three letter
       "codons" which represent the amino acids the proteins encodes in an organism's
       genes. The linear sequence of codons along the DNA molecule is translated into the
       linear sequence of amino acids in the protein(s) encoded by those genes. The code is
       highly degenerate, with 61 codons coding for the 20 natural amino acids and 3 codons
30   representing "stop" signals. Thus, most amino acids are coded for by more than one
       codon - in fact several are coded for by four or more different codons.

       Where more than one codon is available to code for a given amino acid, it has been
       observed that the codon usage patterns of organisms are highly non-random. Different
35   species show a different bias in their codon selection and, furthermore, utilisation of
       codons may be markedly different in a single species between genes which are
       expressed at high and low levels. This bias is different in viruses, plants, bacteria and

5   mammalian cells, and some species show a stronger bias away from a random codon

selection than others. For example, humans and other mammals are less strongly

biased than certain bacteria or viruses. For these reasons, there is a significant

probability that a mammalian gene expressed in E.coli or a viral gene expressed in

mammalian cells will have an inappropriate distribution of codons for efficient

10   expression. It is believed that the presence in a heterologous DNA sequence of

clusters of codons which are rarely observed in the host in which expression is to

occur, is predictive of low heterologous expression levels in that host.

In the polynucleotides of the present invention, the codon usage pattern is altered from

15   that typical of human immunodeficiency viruses to more closely represent the codon

bias of the target organism, e.g. a mammal, especially a human. The "codon usage

coefficient" is a measure of how closely the codon pattern of a given polynucleotide

sequence resembles that of a target species. Codon frequencies can be derived from

literature sources for the highly expressed genes of many species (see e.g. Nakamura

20   et.al. Nucleic Acids Research 1996, 24:214-215). The codon frequencies for each of

the 61 codons (expressed as the number of occurrences occurrence per 1000 codons of

the selected class of genes) are normalised for each of the twenty natural amino acids,

so that the value for the most frequently used codon for each amino acid is set to 1 and

the frequencies for the less common codons are scaled to lie between zero and 1. Thus

25   each of the 61 codons is assigned a value of 1 or lower for the highly expressed genes

of the target species. In order to calculate a codon usage coefficient for a specific

polynucleotide, relative to the highly expressed genes of that species, the scaled value

for each codon of the specific polynucleotide are noted and the geometric mean of all

these values is taken (by dividing the sum of the natural logs of these values by the

30   total number of codons and take the anti-log). The coefficient will have a value

between zero and 1 and the higher the coefficient the more codons in the

polynucleotide are frequently used codons. If a polynucleotide sequence has a codon

usage coefficient of 1, all of the codons are "most frequent" codons for highly

expressed genes of the target species.

35

According to the present invention, the codon usage pattern of the polynucleotide will

preferably exclude rare codons representing less than 10% of the codon use for an

5   amino acid in highly expressed genes of the target organism. In an alternative
       preferred embodiment, the polynucleotide will exclude codons with an RSCU value of
       less than 0.2 in highly expressed genes of the target organism. A relative synonymous
       codon usage (RSCU) value is the observed number of codons divided by the number
       expected if all codons for that amino acid were used equally frequently. A

10   polynucleotide of the present invention will generally have a codon usage coefficient
       for highly expressed human genes of greater than 0.3, preferably greater than 0.4, most
       preferably greater than 0.5. Codon usage tables for human can also be found in
       Genebank.

15   In comparison, a highly expressed beta action gene has a RSCU of 0.747. The codon
       usage table for a *homo sapiens* is set out below:

       Codon Usage Table:

20   **Homo sapiens [gbpri]: 27143 CDS's (12816923 codons) Standard Codon Usage
       Table**

       fields: [triplet] [frequency: **per thousand**] ([number])

25   UUU 17.0(217684) UCU 14.8(189419) UAU 12.1(155645) UGU 10.0(127719)
       UUC 20.5(262753) UCC 17.5(224470) UAC 15.8(202481) UGC 12.3(157257)
       UUA 7.3(93924) UCA 11.9(152074) UAA 0.7(9195) UGA 1.3(16025)
       UUG 12.5(159611) UCG 4.5(57572) UAG 0.5(6789) UGG 12.9(165930)

30   CUU 12.8(163707) CCU 17.3(222146) CAU 10.5(134186) CGU 4.6(59454)
       CUC 19.3(247391) CCC 20.0(256235) CAC 14.9(190928) CGC 10.8(137865)
       CUA 7.0(89078) CCA 16.7(214583) CAA 12.0(153590) CGA 6.3(80709)
       CUG 39.7(509096) CCG 7.0(89619) CAG 34.5(441727) CGG 11.6(148666)

35   AUU 15.8(202844) ACU 12.9(165392) AAU 17.0(218508) AGU 12.0(154442)
       AUC 21.6(277066) ACC 19.3(247805) AAC 19.8(253475) AGC 19.3(247583)
       AUA 7.2(92133) ACA 14.9(191518) AAA 24.0(308123) AGA 11.5(147264)
       AUG 22.3(285776) ACG 6.3(80369) AAG 32.6(418141) AGG 11.3(145276)

40   GUU 10.9(139611) GCU 18.5(236639) GAU 22.4(286742) GGU 10.8(138606)
       GUC 14.6(187333) GCC 28.3(362086) GAC 26.1(334158) GGC 22.7(290904)
       GUA 7.0(89644) GCA 15.9(203310) GAA 29.1(373151) GGA 16.4(210643)
       GUG 28.8(369006) GCG 7.5(96455) GAG 40.2(515485) GGG 16.4(209907)

17

5   Coding GC 52.51% 1st letter GC 56.04% 2nd letter GC 42.35% 3rd letter GC
      59.13%.

      According to a further aspect of the invention, an expression vector is provided which
      comprises and is capable of directing the expression of a Nef and/or Tat or NefTat,
10   and a gp120 polynucleotide sequence according to the first aspect of the invention, in
      particular where the codon usage pattern of at least one of the Nef, Tat, NefTat or
      gp120 polynucleotide sequences, particularly the gp120 sequence, is typical of highly
      expressed mammalian genes, preferably highly expressed human genes. The vector is
      suitable for driving expression of heterologous DNA in mammalian cells, particularly
15   human cells. In one embodiment, the expression vector is p7313 (see figure 22).

      In a further aspect the invention provides a plurality of particles, preferably gold
      particles, coated with DNA comprising one or more vectors encoding gp120 and nef
      and/or tat or neftat. Preferably the particles are coated with a single vector which
20   encodes gp120 and nef and tat, the latter most preferably in the form of a NefTat
      fusion protein. Most preferably one or more of the sequences are codon optimised for
      expression in human cells.

      In a preferred aspect the DNA encoding the nef, tat and gp 120 is present on a single
25   vector.

      Preferably the vector comprises the nef, tat and gp 120 sequences inserted 3' to an
      enhanced HCMV IE1 promoter for efficient expression. This is preferably the HCMV
      immediate early promoter devoid of intron A, but including exon 1.

30

      One suitable vector according to the invention is that denoted as p7313, further
      described below.

      The vectors which comprise the nucleotide sequences described herein are
35   administered in such amount as will be prophylactically or therapeutically effective.
      The quantity to be administered is generally in the range of one picogram to 1
      milligram, preferably 1 picogram to 10 micrograms for particle-mediated delivery as

described herein. The exact quantity may vary depending on the weight of the patient being immunised and the precise route of administration.

Immunisations according to the invention may be performed with a combination of protein and DNA-based formulations. Adjuvanted protein vaccines induce mainly antibodies and T helper immune responses, while delivery of DNA as a plasmid or a live vector induces strong cytotoxic T lymphocyte (CTL) responses. Thus, the combination of protein and DNA vaccination will provide for a wide variety of immune responses. This is particularly relevant in the context of HIV, since both neutralising antibodies and CTL are thought to be important for the immune defence against HIV.

The DNA may be delivered as plasmid DNA or in the form of a recombinant live vector, e.g. a poxvirus vector or any other suitable live vector such as those described herein. Protein antigens may be injected once or several times followed by one or more DNA administrations, or DNA may be used first for one or more administrations followed by one or more protein immunisations.

A particular example of prime-boost immunisation according to the invention involves priming with DNA in the form of a recombinant live vector such as a modified poxvirus vector, for example Modified Virus Ankara (MVA) or a derivative thereof e.g through passaging or genetic manipulation, or an alphavirus vecotr for example Venezuelian Equine Encephalitis Virus, followed by boosting with a protein, preferably an adjuvanted protein. Optionally the DNA is adjuvanted with a suitable DNA vaccine adjuvant known in the art.

The invention further provides a method of prophylactic or therapeutic immunisation which method comprises administering to a subject in need thereof a composition comprising

a) an HIV Tat protein or polynucleotide: or

b) an HIV Nef protein or polynucleotide; or

c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide;

5   and an HIV gp120 protein or polynucleotide, in a prime-boost delivery wherein the
protein or polynucleotide is delivered via a bombardment approach.

In a further aspect the invention provides a kit comprising at least two different
vaccine compositions including a) a composition comprising a plurality of particles
10   coated with DNA encoding gp120 and nef and/or tat or neftat as described herein and
b) a composition comprising gp120 and nef and/or tat or neftat DNA or proteins as
described herein wherein the DNA or proteins in b) are not coated onto particles.

The invention is illustrated in the accompanying examples and Figures:
15

EXAMPLES

General

20

The Nef gene from the Bru/Lai isolate (Cell 40: 9-17, 1985) was selected for
the constructs of these experiments since this gene is among those that are most
closely related to the consensus Nef.

The starting material for the Bru/Lai Nef gene was a 1170bp DNA fragment
25   cloned on the mammalian expression vector pcDNA3 (pcDNA3/Nef).

The Tat gene originates from the BH10 molecular clone. This gene was
received as an HTLV III cDNA clone named pCV1 and described in Science, 229,
p69-73, 1985.

30   The expression of the Nef and Tat genes could be in Pichia or any other host.

Example 1.   EXPRESSION OF HIV-1 *nef* AND *tat* SEQUENCES IN PICHIA
35   **PASTORIS.**

5　　Nef protein, Tat protein and the fusion Nef -Tat were expressed in the methylotrophic

yeast *Pichia pastoris* under the control of the inducible alcohol oxidase (AOX1)

promoter.

To express these HIV-1 genes a modified version of the integrative vector PHIL-D2

10　　(INVITROGEN) was used. This vector was modified in such a way that expression of

heterologous protein starts immediately after the native ATG codon of the AOX1 gene

and will produce recombinant protein with a tail of one glycine and six histidines

residues . This PHIL-D2-MOD vector was constructed by cloning an oligonucleotide

linker between the adjacent AsuII and EcoRI sites of PHIL-D2 vector (see Figure 2

15　　which includes Seq ID Nos: 26 and 27). In addition to the His tail, this linker carries

NcoI, SpeI and XbaI restriction sites between which *nef, tat* and *nef-tat* fusion were

inserted.

1.1　　CONSTRUCTION OF THE INTEGRATIVE VECTORS pRIT14597

20　　(encoding Nef-His protein), pRIT14598 (encoding Tat-His protein) and

pRIT14599 (encoding fusion Nef-Tat-His).

The *nef* gene was amplified by PCR from the pcDNA3/Nef plasmid with primers 01

and 02.

25

NcoI

PRIMER 01 (Seq ID NO 1): 5'ATCGT<u>CCATG.G</u>GT.GGC.AAG.TGG.T 3'

SpeI

30　　PRIMER 02 (Seq ID NO 2): 5' CGGCT<u>ACTAGT</u>GCAGTTCTTGAA 3'

The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both

restricted by NcoI and SpeI, purified on agarose gel and ligated to create the

integrative plasmid pRIT14597 (see Figure 2).

35

The *tat* gene was amplified by PCR from a derivative of the pCV1 plasmid

with primers 05 and 04:

5   SpeI

PRIMER 04 (Seq ID NO 4): 5' CGGCTA<u>CTAGT</u>TTCCTTCGGGCCT 3'

                                           NcoI

10   PRIMER 05 (Seq ID NO 5): 5'ATCGT<u>CCATGG</u>AGCCAGTAGATC 3'

An NcoI restriction site was introduced at the 5' end of the PCR fragment while a
SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and
the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose

15   gel and ligated to create the integrative plasmid pRIT14598.

To construct pRIT14599, a 910bp DNA fragment corresponding to the *nef-tat*-His
coding sequence was ligated between the EcoRI blunted(T4 polymerase)
and NcoI sites of the PHIL-D2-MOD vector. The *nef-tat*-His coding fragment was

20   obtained by XbaI blunted(T4 polymerase) and NcoI digestions of pRIT14596.

1.2   TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115(his4).

To obtain *Pichia pastoris* strains expressing Nef-His, Tat-His and the fusion Nef-Tat-

25   His, strain GS115 was transformed with linear NotI fragments carrying the respective
expression cassettes plus the HIS4 gene to complement his4 in the host
genome.Transformation of GS115 with NotI-linear fragments favors recombination at
the AOXI locus.

30   Multicopy integrant clones were selected by quantitative dot blot analysis and the type
of integration, insertion (Mut[+]phenotype) or transplacement (Mut[s]phenotype),
was determined.

From each transformation, one transformant showing a high production level for the

35   recombinant protein was selected :

Strain Y1738 (Mut[+] phenotype) producing the recombinant Nef-His protein,

5   a myristylated 215 amino acids protein which is composed of:

°Myristic acid

°A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD

vector

10   °205 a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)

°A threonine and a serine created by the cloning procedure (cloning at SpeI

site of PHIL-D2-MOD vector.

°One glycine and six histidines.

15   Strain Y1739 (Mut$^+$ phenotype) producing the Tat-His protein, a 95 amino acid

protein which is composed of:

°A methionine created by the use of NcoI cloning site

°85 a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)

20

°A threonine and a serine introduced by cloning procedure

°One glycine and six histidines

Strain Y1737(Mut^s phenotype) producing the recombinant Nef-Tat-His fusion protein,

25   a myristylated 302 amino acids protein which is composed of:

°Myristic acid

°A methionine, created by the use of NcoI cloning site

°205a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)

30   °A threonine and a serine created by the cloning procedure

°85a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)

°A threonine and a serine introduced by the cloning procedure

°One glycine and six histidines

35

Example 2.   EXPRESSION OF HIV-1 Tat-MUTANT IN PICHIA PASTORIS

5   A mutant recombinant Tat protein has also been expressed. The mutant Tat protein
     must be **biologically inactive** while **maintaining** its **immunogenic epitopes.**

     A double mutant *tat* gene, constructed by D.Clements (Tulane University) was
     selected for these constructs.

10

     This *tat* gene (originates from BH10 molecular clone) bears **mutations in the active
     site region (Lys41→Ala)and in RGD motif (Arg78→Lys and Asp80→Glu)**
     (Virology 235: 48-64, 1997).

15   The mutant *tat* gene was received as a cDNA fragment subcloned between the EcoRI
     and HindIII sites within a CMV expression plasmid (pCMVLys41/KGE)

     2.1   CONSTRUCTION OF THE INTEGRATIVE VECTORS
20   **pRIT14912(encoding Tat mutant-His protein) and pRIT14913(encoding fusion
     Nef-Tat mutant-His).**

     The *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with
     primers 05 and 04 (see section 1.1construction of pRIT14598)

25

     An NcoI restriction site was introduced at the 5' end of the PCR fragment while a
     SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and
     the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose
     gel and ligated to create the integrative plasmid pRIT14912

30

     To construct pRIT14913, the *tat* mutant gene was amplified by PCR from the
     pCMVLys41/KGE plasmid with primers 03 and 04.

                                        SpeI

35   PRIMER 03 (Seq ID NO 3): 5' ATCGT<u>ACTAGT</u>.GAG.CCA.GTA.GAT.C 3'

                                        SpeI

5   PRIMER 04 (Seq ID NO 4): 5' CGGCT<u>ACTAGT</u>TTCCTTCGGGCCT 3'

The PCR fragment obtained and the plasmid pRIT14597 (expressing Nef-His protein) were both digested by SpeI restriction enzyme, purified on agarose gel and ligated to

10   create the integrative plasmid pRIT14913

2.2   TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115.

<u>Pichia pastoris</u> strains expressing Tat mutant-His protein and the fusion Nef-Tat

15   mutant-His were obtained, by applying integration and recombinant strain selection strategies previously described in section 1.2 .

Two recombinant strains producing Tat mutant-His protein ,a 95 amino-acids protein, were selected: Y1775 (Mut$^+$ phenotype) and Y1776(Mut^s phenotype).

20

One recombinant strain expressing Nef-Tat mutant-His fusion protein, a 302 amino-acids protein was selected: Y1774(Mut$^+$ phenotype).

25

Example 3: FERMENTATION OF PICHIA PASTORIS PRODUCING RECOMBINANT TAT-HIS.

A typical process is described in the table hereafter.

30

Fermentation includes a growth phase (feeding with a glycerol-based medium according to an appropriate curve) leading to a high cell density culture and an induction phase (feeding with a methanol and a salts/micro-elements solution). During fermentation the growth is followed by taking samples and measuring their

35   absorbance at 620 nm. During the induction phase methanol was added via a pump and its concentration monitored by Gas chromatography (on culture samples) and by on-line gas analysis with a Mass spectrometer. After fermentation the cells were

5   recovered by centrifugation at 5020g during 30' at 2-8°C and the cell paste stored at –

20°C. For further work cell paste was thawed, resuspended at an OD (at 620 nm) of

150 in a buffer (Na2HPO4 pH7 50 mM, PMSF 5%, Isopropanol 4 mM) and disrupted

by 4 passages in a DynoMill (room 0.6L, 3000 rpm, 6L/H, beads diameter of 0.40-

0.70 mm).

10   For evaluation of the expression samples were removed during the induction,

disrupted and analyzed by SDS-Page or Western blot. On Coomassie blue stained

SDS-gels the recombinant Tat-his was clearly identified as an intense band presenting

a maximal intensity after around 72-96H induction.

5

Thawing of a Working seed vial	
↓	
Solid preculture 30°C, 14-16H	Synthetic medium: YNB + glucose + agar
↓	
Liquid preculture in two 2L erlenmeyer 30°C, 200 rpm	Synthetic medium: 2 x 400 ml YNB + glycerol Stop when OD > 1 (at 620 nm)
↓	
Inoculation of a 20L fermentor	5L initial medium (FSC006AA) 3 ml antifoam SAG471 (from Witco) Set-points: Temperature : 30°C Overpressure: 0.3 barg Air flow: 20 Nl/min Dissolved O2: regulated > 40% pH : regulated at 5 by NH_4OH
↓	
Fed-batch fermentation: growth phase Duration around 40H	Feeding with glycerol-based medium FFB005AA Final OD between 200-500 OD (620 nm)
Fed-batch fermentation: induction phase Duration: up to 97H	Feeding with methanol and with a salt/micro- elements solution (FSE021AB).
↓	
Centrifugation	5020g /30 min / 2-8°C
↓	
Recover cell paste and store at –20°C	
↓	
Thaw cells and resuspend at OD150 (620 nm) in buffer	Buffer: Na2HPO4 pH7 50 mM, PMSF 5%, Isopropanol 4 mM
↓	
Cell disruption in Dyno-mill 4 passages	Dyno-mill: (room 0.6L, 3000 rpm, 6L/H, beads diameter of 0.40-0.70 mm).
↓	
Transfer for extraction/purification	

5   **Media used for fermentation:**

Solid preculture: (YNB + glucose + agar)

Glucose:	10 g/l	Na2MoO4.2H2O:	0.0002 g/l	Acide folique:	
KH2PO4:	1 g/l	MnSO4.H2O:	0.0004 g/l	Inositol:	0.064 g/l
MgSO4.7H2O:	0.5 g/l	H3BO3:	0.0005 g/l	Pyridoxine:	0.008 g/l
CaCl2.2H2O:	0.1 g/l	KI:	0.0001 g/l	Thiamine:	0.008 g/l
NaCl:	0.1 g/l	CoCl2.6H2O:	0.00009 g/l	Niacine:	0.000032 g/l
FeCl3.6H2O:	0.0002 g/l	Riboflavine:	0.000016 g/l	Panthoténate Ca:	0.008 g/l
CuSO4.5H2O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid:	
ZnSO4.7H2O:	0.0004 g/l	(NH4)2SO4:	5 g/l	Agar	18 g/l

Liquid preculture ,(YNB + glycerol)

Glycerol:	2% (v/v)	Na2MoO4.2H2O:	0.0002 g/l	Acide folique:	
KH2PO4:	1 g/l	MnSO4.H2O:	0.0004 g/l	Inositol:	0.064 g/l
MgSO4.7H2O:	0.5 g/l	H3BO3:	0.0005 g/l	Pyridoxine:	0.008 g/l
CaCl2.2H2O:	0.1 g/l	KI:	0.0001 g/l	Thiamine:	0.008 g/l
NaCl:	0.1 g/l	CoCl2.6H2O:	0.00009 g/l	Niacine:	0.000032 g/l
FeCl3.6H2O:	0.0002 g/l	Riboflavine:	0.000016 g/l	Panthoténate Ca:	0.008 g/l
CuSO4.5H2O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid:	
ZnSO4.7H2O:	0.0004 g/l	(NH4)2SO4:	5 g/l		

Initial fermentor charge: (FSC006AA)

10

$(NH4)_2SO4$:	6.4 g/l			
KH2PO4:	9 g/l	Na2MoO4.2H2O:	2.04 mg/l	
MgSO4.7H2O:		4.7 g/l	MnSO4.H2O:	4.08 mg/l
CaCl2.2H2O:	0.94 g/l	H3BO3:	5.1 mg/l	
FeCl3.6H2O:	10 mg/l	KI:	1.022 mg/l	
HCl:	1.67 ml/l	CoCl2.6H2O:	0.91mg/l	
CuSO4.5H2O:	0.408 mg/l	NaCl:	0.06 g/l	
ZnSO4.7H2O:	4.08 mg/l	Biotine:	0.534 mg/l	

15

20

5

Feeding solution used for growth phase (FFB005AA)			
Glycérol:	38.7 % v/v mg/l	Na2MoO4.2H2O:	5.7
MgSO4.7H2O:	13 g/l	CuSO4.5H2O:	1.13 mg/l
CaCl2.2H2O:	2.6 g/l	CoCl2.6H2O:	2.5 mg/l
FeCl3.6H2O:	27.8mg/l	H3BO3:	14.2 mg/l
ZnSO4.7H2O	11.3 mg/l	Biotine:	1.5 mg/l
MnSO4.H2O:	11.3 mg/l	KI:	2.84mg/l
KH2PO4:	24.93 g/l	NaCl:	0.167 g/l

Feeding solution of salts and micro-elements used during induction (FSE021AB):				
KH2PO4:	45 g/l	Na2MoO4.2H2O:	10.2 mg/l	
MgSO4.7H2O:	mg/l	23.5 g/l	MnSO4.H2O:	20.4
CaCl2.2H2O:	4.70 g/l	H3BO3:	25.5 mg/l	
NaCl:	0.3 g/l	KI:	5.11 mg/l	
HCl:	8.3 ml/l	CoCl2.6H2O:	4.55mg/l	
CuSO4.5H2O:	mg/l	2.04 mg/l	FeCl3.6H2O:	50.0
ZnSO4.7H2O:20.4 mg/l		Biotine:	2.70 mg/l	

30

Example 4:   PURIFICATION OF Nef-Tat-His FUSION PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 146g of recombinant Pichia pastoris cells (wet weight) or 2L Dyno-mill homogenate OD 55. The chromatographic steps are performed at room temperature. Between steps , Nef-Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

146g of Pichia pastoris cells

↓

Homogenization   Buffer: 2L 50 mM PO₄ pH 7.0

final OD:50

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation   JA10 rotor / 9500 rpm/ 30 min /

                                                        room temperature

↓

Dyno-mill Pellet

↓

Wash   Buffer: +2L 10 mM PO_4 pH 7.5 -

(1h - 4°C)   150mM - NaCl 0,5% empigen

↓

Centrifugation   JA10 rotor / 9500 rpm/ 30 min /

                                                        room temperature

↓

Pellet

↓

Solubilisation   Buffer: + 660ml 10 mM PO_4 pH

(O/N - 4°C)   7.5 - 150mM NaCl - 4.0M GuHCl

↓

Reduction   + 0,2M 2-mercaptoethanesulfonic

(4H – room temperature - in the dark)   acid, sodium salt (powder

                                                        addition) / pH adjusted to 7.5

                                                        (with 0,5M NaOH solution) before

                                                        incubation

↓

reaction with iodoacetamide   + 0,25M Iodoacetamide (powder

(carbamidomethylation)   addition) / pH adjusted to 7.5

(1/2 h – room temperature - in the dark)   (with 0,5M NaOH solution) before

                                                        incubation

↓

Immobilized metal ion affinity   Equilibration buffer: 10 mM PO_4

chromatography on Ni^{++}-NTA-Agarose   pH 7.5 - 150mM NaCl - 4.0M

(Qiagen - 30 ml of resin)

GuHCl

Washing buffer: 1) Equilibration buffer

2) 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea

3) 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea - 25 mM   Imidazol

Elution buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea - 0,5M Imidazol

↓

Dilution

Down to an ionic strength of 18 mS/cm²

Dilution buffer: 10 mM PO_4 pH 7.5 - 6M Urea

↓

Cation exchange chromatography on SP Sepharose FF
 (Pharmacia - 30 ml of resin)

Equilibration buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6.0M Urea

Washing buffer: 1) Equilibration buffer

2) 10 mM PO_4 pH 7.5 - 250mM NaCl - 6M Urea

Elution buffer: 10 mM Borate pH 9.0 - 2M NaCl - 6M Urea

↓

Concentration

up to 5 mg/ml

10kDa Omega membrane(Filtron)

↓

Gel filtration chromatography on Superdex200 XK 16/60

Elution buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea

(Pharmacia - 120 ml of resin)   5 ml of sample / injection ➜ 5
                                                 injections

↓

Dialysis   Buffer: 10 mM PO$_4$ pH 6.8 -

(O/N - 4°C)   150mM NaCl -   0,5M Arginin*

↓

Sterile filtration   Millex GV 0,22μm

* ratio: 0,5M Arginin for a protein concentration of 1600μg/ml.

Purity

The level of purity as estimated by SDS-PAGE is shown in Figure 3 by Daiichi Silver
Staining and in Figure 4 by Coomassie blue G250.

After Superdex200 step:	> 95%
After dialysis and sterile filtration steps:	> 95%

Recovery

51mg of Nef-Tat-his protein are purified from 146g of recombinant Pichia pastoris
cells (= 2L of Dyno-mill homogenate OD 55)

**Example 5:   PURIFICATION OF OXIDIZED NEF-TAT-HIS FUSION
PROTEIN IN PICHIA PASTORIS**

The purification scheme has been developed from 73 g of recombinant Pichia pastoris
cells (wet weight) or 1 L Dyno-mill homogenate OD 50. The chromatographic steps
are performed at room temperature. Between steps , Nef-Tat positive fractions are
kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

5

73 g of Pichia pastoris cells

↓

Homogenization
                                      Buffer: 1L 50 mM PO_4 pH 7.0 –

Pefabloc 5 mM

final OD:50

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation
                                        JA10 rotor / 9500 rpm/ 30 min / room

temperature

↓

Dyno-mill Pellet

↓

Wash
                                            Buffer: +1L 10 mM PO_4 pH 7.5 – 150

(2h - 4°C)
                                        mM NaCl - 0,5% Empigen

↓

Centrifugation
                                        JA10 rotor / 9500 rpm/ 30 min / room

temperature

↓

Pellet

↓

Solubilisation
                                        Buffer: + 330ml 10 mM PO_4 pH 7.5 -

(O/N - 4°C)
                                        150mM NaCl - 4.0M GuHCl

↓

Immobilized metal ion affinity
                                      Equilibration buffer: 10 mM PO_4 pH 7.5

chromatography on Ni^{++}-NTA-Agarose
                       – 150 mM NaCl - 4.0 M GuHCl

(Qiagen - 15 ml of resin)
                                      Washing buffer: 1) Equilibration buffer

                                                    2) 10 mM PO_4 pH 7.5

                       –   150 mM NaCl – 6 M

Urea

                                                  3) 10 mM PO_4 pH 7.5

	— 150 mM NaCl – 6 M Urea - 25 mM Imidazol

Elution buffer: 10 mM PO$_4$ pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol

↓

Dilution

Down to an ionic strength of 18 mS/cm^2

Dilution buffer: 10 mM PO$_4$ pH 7.5 – 6 M Urea

↓

Cation exchange chromatography on SP Sepharose FF

(Pharmacia - 7 ml of resin)

Equilibration buffer: 10 mM PO$_4$ pH 7.5 – 150 mM NaCl - 6.0 M Urea

Washing buffer: 1) Equilibration buffer

2) 10 mM PO$_4$ pH 7.5 – 250 mM NaCl – 6 M Urea

Elution buffer: 10 mM Borate pH 9.0 – 2 M NaCl – 6 M Urea

↓

Concentration

up to 0,8 mg/ml

10kDa Omega membrane(Filtron)

↓

Dialysis

(O/N - 4°C)

↓

Buffer: 10 mM PO$_4$ pH 6.8 – 150 mM NaCl – 0,5 M Arginin

Sterile filtration

Millex GV 0,22μm

→ Level of purity estimated by SDS-PAGE is shown in Figure 6 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

5

➔ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

2,8 mg of oxidized Nef-Tat-his protein are purified from 73 g of recombinant Pichia pastoris cells (wet weight) or 1 L of Dyno-mill homogenate OD 50.

10

Example 6: PURIFICATION OF REDUCED TAT-HIS PROTEIN (PICHIA PASTORIS)

15

The purification scheme has been developed from 160 g of recombinant Pichia pastoris cells (wet weight) or 2L Dyno-mill homogenate OD 66. The chromatographic steps are performed at room temperature. Between steps, Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time,

20   samples are frozen at -20°C.

160 g of Pichia pastoris cells
↓
Homogenization   Buffer: +2 L   50 mM PO_4 pH 7.0 – 4 mM PMSF
                                              final OD:66

↓

Dyno-mill disruption (4 passes)
↓
Centrifugation   JA10 rotor / 9500 rpm / 30 min / room temperature
↓
Dyno-mill Pellet
↓
Wash   Buffer: +2 L 10 mM PO_4 pH 7.5 – 150 mM NaCl
(1h - 4°C)   - 1% Empigen
↓

Centrifugation   JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Pellet

↓

Solubilisation   Buffer: + 660 ml 10 mM PO_4 pH 7.5 – 150 mM
(O/N - 4°C)   NaCl - 4.0 M GuHCl

↓

Centrifugation   JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Reduction   + 0,2 M 2-mercaptoethanesulfonic acid, sodium
(4H - room temperature - in the dark)   salt (powder addition) / pH adjusted to 7.5 (with
                                        1 M NaOH solution) before incubation

↓

reaction with iodoacetamide   + 0,25 M Iodoacetamide (powder addition) / pH
(1/2 h - room temperature - in the dark) adjusted to 7.5 (with 1 M NaOH solution) before
                                        incubation

↓

Immobilized metal ion affinity   Equilibration buffer: 10 mM PO_4 pH 7.5 – 150 mM
chromatography on Ni^{++}-NTA-Agarose   NaCl - 4.0 M GuHCl
(Qiagen - 60 ml of resin)   Washing buffer: 1) Equilibration buffer
                                                        2) 10 mM PO_4 pH 7.5 – 150 mM
                                                        NaCl – 6 M Urea
                                                        3) 10 mM PO_4 pH 7.5 – 150 mM
                                                        NaCl - 6M Urea - 35 mM
                                                        Imidazol
                                        Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl
                                                        – 6 M Urea - 0,5 M Imidazol

↓

Dilution   Down to an ionic strength of 12 mS/cm
                                        Dilution buffer: 20 mM Borate pH 8.5 – 6 M Urea

↓

Cation exchange chromatography on SP   Equilibration buffer: 20 mM Borate pH 8.5 -

Sepharose FF   150 mM NaCl - 6.0 M Urea

(Pharmacia - 30 ml of resin)   <u>Washing buffer</u>: Equilibration buffer

<u>Elution buffer</u>: 20 mM Borate pH 8.5 – 400 mM

NaCl - 6.0 M Urea

↓

Concentration   up to 1,5 mg/ml

10kDa Omega membrane(Filtron)

↓

Dialysis   <u>Buffer</u>: 10 mM PO_4 pH 6.8 – 150 mM NaCl -

(O/N - 4°C)   0,5 M Arginin

↓

Sterile filtration   Millex GV 0,22 μm

→ <u>Level of purity estimated by SDS-PAGE as shown in Figure 7(Daiichi Silver
Staining, Coomassie blue G250, Western blotting):</u>

After dialysis and sterile filtration steps: > 95%

→ <u>Recovery</u> (evaluated by a colorimetric protein assay: DOC TCA BCA)

48 mg of reduced Tat-his protein are purified from 160 g of recombinant
Pichia   pastoris cells (wet weight) or 2 L of
Dyno-mill homogenate OD 66.

Example 7: Purification of oxidized Tat-his protein (Pichia Pastoris)

5   The purification scheme has been developed from 74 g of recombinant Pichia pastoris
     cells (wet weight) or 1L Dyno-mill homogenate OD60. The chromatographic steps
     are performed at room temperature. Between steps, Tat positive fractions are kept
     overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

74 g of Pichia pastoris cells
↓

Homogenization   Buffer: +1 L 50 mM PO_4 pH 7.0 – 5 mM Pefabloc
                                            final OD:60

↓

Dyno-mill disruption (4 passes)
↓

Centrifugation   JA10 rotor / 9500 rpm / 30 min / room temperature
↓

Dyno-mill Pellet
↓

Wash   Buffer:+1 L 10 mM PO_4 pH 7.5 – 150 mM NaCl
(1h - 4°C)   - 1% Empigen
↓

Centrifugation   JA10 rotor / 9500 rpm / 30 min / room temperature
↓

Pellet
↓

Solubilisation   Buffer: + 330 ml 10 mM PO_4 pH 7.5 – 150 mM
(O/N - 4°C)   NaCl - 4.0 M GuHCl
↓

Centrifugation   JA10 rotor / 9500 rpm / 30 min / room temperature
↓

Immobilized metal ion affinity   Equilibration buffer: 10 mM PO_4 pH 7.5 –150 mM

chromatography on Ni^{++}-NTA-Agarose

(Qiagen - 30 ml of resin)

NaCl - 4.0 M GuHCl

<u>Washing buffer</u>: 1) Equilibration buffer

2) 10 mM PO_4 pH 7.5 – 150 mM

NaCl – 6 M Urea

3) 10 mM PO_4 pH 7.5 – 150 mM

NaCl – 6 M Urea - 35 mM

Imidazol

<u>Elution buffer</u>: 10 mM PO_4 pH 7.5 – 150 mM

NaCl – 6 M Urea - 0,5 M Imidazol

↓

Dilution

Down to an ionic strength of 12 mS/cm

<u>Dilution buffer</u>: 20 mM Borate pH 8.5 – 6 M Urea

↓

Cation exchange chromatography on SP
Sepharose FF

(Pharmacia - 15 ml of resin)

<u>Equilibration buffer</u>: 20 mM Borate pH 8.5 -

150 mM NaCl - 6.0 M Urea

<u>Washing buffer</u>: 1) Equilibration buffer

2) 20 mM Borate pH 8.5 -

400 mM NaCl - 6.0 M Urea

<u>Elution buffer</u>: 20 mM Piperazine pH 11.0 – 2 M

NaCl – 6 M Urea

↓

Concentration

up to 1,5 mg/ml

10 kDa Omega membrane(Filtron)

↓

Dialysis

(O/N - 4°C)

<u>Buffer</u>: 10 mM PO_4 pH 6.8 – 150 mM NaCl -

0,5 M Arginin

↓

Sterile filtration

Millex GV 0,22 μm

5

→ <u>Level of purity estimated by SDS-PAGE as shown in Figure 8 (Daiichi Silver
Staining, Coomassie blue G250, Western blotting)</u>:

5   After dialysis and sterile filtration steps: > 95%

→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

        19 mg of oxidized Tat-his protein are purified from 74 g of recombinant

10 Pichia   pastoris cells (wet weight) or 1 L of

Dyno-mill homogenate OD 60.

Example 8: PURIFICATION OF SIV REDUCED NEF-HIS PROTEIN

15 **(PICHIA PASTORIS)**

The purification scheme has been developed from 340 g of recombinant Pichia

pastoris cells (wet weight) or 4 L Dyno-mill homogenate OD 100. The

20 chromatographic steps are performed at room temperature. Between steps , Nef

positive fractions are kept overnight in the cold room (+4°C) ; for longer time,

samples are frozen at -20°C.

25

340 g of Pichia pastoris cells

↓

Homogenization   Buffer: 4L 50 mM PO_4 pH 7.0 – PMSF

                                     4 mM

                                     final OD:100

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation   JA10 rotor / 9500 rpm/ 60 min / room

                                     temperature

↓

Dyno-mill Pellet

↓

Solubilisation

(O/N - 4°C)

$\underline{Buffer}$: + 2,6 L 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl

↓

Centrifugation

JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Reduction

(4H - room temperature - in the dark)

+ 0,2 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation

↓

Reaction with iodoacetamide

(1/2 h - room temperature - in the dark)

+ 0,25 M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation

↓

Immobilized metal ion affinity

chromatography on Ni^{++}-NTA-Agarose

(Qiagen - 40 ml of resin)

$\underline{Equilibration\ buffer}$: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl

$\underline{Washing\ buffer}$:   1) Equilibration buffer

                              2) 10 mM PO_4 pH 7.5

–   150 mM NaCl – 6 M

Urea -   25 mM Imidazol

$\underline{Elution\ buffer}$: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol

↓

Concentration

up to 3 mg/ml

10kDa Omega membrane(Filtron)

↓

Gel filtration chromatography on

Superdex 200

$\underline{Elution\ buffer}$: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea

(Pharmacia - 120 ml of resin)

↓

Concentration   up to 1,5 mg/ml

                                               10kDa Omega membrane(Filtron)

↓

Dialysis   <u>Buffer</u>: 10 mM PO$_4$ pH 6.8 – 150 mM

(O/N - 4°C)   NaCl – Empigen 0,3%

↓

Sterile filtration   Millex GV 0,22μm

5

→ <u>Level of purity estimated by SDS-PAGE as shown in Figure 9 (Daiichi Silver Staining, Coomassie blue G250, Western blotting)</u>:

        After dialysis and sterile filtration steps: > 95%

10

→ <u>Recovery</u> (evaluated by a colorimetric protein assay: DOC TCA BCA)

20 mg of SIV reduced Nef -his protein are purified from 340 g of recombinant Pichia

15 pastoris cells (wet weight) or 4 L of Dyno-mill homogenate OD 100.

Example 9: PURIFICATION OF HIV REDUCED NEF-HIS PROTEIN (PICHIA PASTORIS)

20

The purification scheme has been developed from 160 g of recombinant Pichia pastoris cells (wet weight) or 3 L Dyno-mill homogenate OD 50. The chromatographic steps are performed at room temperature. Between steps , Nef

25 positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

5

160 g of Pichia pastoris cells

↓

Homogenization   <u>Buffer</u>: 3 L 50 mM PO_4 pH 7.0 –

Pefabloc 5 mM final OD:50

↓

Dyno-mill disruption (4 passes)

↓

Freezing/Thawing

↓

Centrifugation   JA10 rotor / 9500 rpm/ 60 min / room

temperature

↓

Dyno-mill Pellet

↓

Solubilisation   <u>Buffer</u>: + 1 L 10 mM PO_4 pH 7.5 -

(O/N - 4°C)   150mM NaCl - 4.0M GuHCl

↓

Centrifugation   JA10 rotor / 9500 rpm / 60 min / room

temperature

↓

Reduction   + 0,1 M 2-mercaptoethanesulfonic acid,

(3 H - room temperature - in the dark)   sodium salt (powder addition) / pH

adjusted to 7.5 (with   1 M NaOH

solution) before incubation

↓

Reaction with iodoacetamide   + 0,15 M Iodoacetamide (powder

(1/2 h - room temperature - in the dark)   addition) / pH adjusted to 7.5 (with 1

M NaOH solution) before incubation

↓

Immobilized metal ion affinity   <u>Equilibration buffer</u>: 10 mM PO_4 pH 7.5

chromatography on Ni^{++}-NTA-Agarose   – 150 mM NaCl - 4.0 M GuHCl

(Qiagen - 10 ml of resin)   Washing buffer: 1) Equilibration buffer

                                                      2) 10 mM PO$_4$ pH 7.5

               –   150 mM NaCl – 6 M

Urea

                                                      3) 10 mM PO$_4$ pH 7.5

               –   150 mM NaCl – 6 M

Urea -   25 mM Imidazol

Elution buffer: 10 mM Citrate pH 6.0 -

150 mM NaCl – 6 M Urea - 0,5 M

Imidazol

↓

Concentration   up to 3 mg/ml

                                              10kDa Omega membrane(Filtron)

↓

Gel filtration chromatography on   Elution buffer: 10 mM PO$_4$ pH 7.5 –

Superdex 200   150 mM NaCl – 6 M Urea

(Pharmacia - 120 ml of resin)

↓

Dialysis   Buffer: 10 mM PO$_4$ pH 6.8 – 150 mM

(O/N - 4°C)   NaCl – 0,5M Arginin

↓

Sterile filtration   Millex GV 0,22μm

5

➔ Level of purity estimated by SDS-PAGE as shown in Figure 10 (Daiichi Silver
Staining, Coomassie blue G250, Western blotting):

       After dialysis and sterile filtration steps: > 95%

10

➔ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

5   20 mg of HIV reduced Nef-his protein are purified from 160 g of recombinant
Pichia pastoris cells (wet weight) or 3 L of Dyno-mill homogenate OD 50.

Example 10: EXPRESSION OF SIV *nef* SEQUENCE IN *PICHIA PASTORIS*

10

In order to evaluate Nef and Tat antigens in the pathogenic SHIV challenge model, we
have expressed the Nef protein of simian immunodeficiency virus (SIV) of macaques,
SIVmac239 (Aids Research and Human Retroviruses, 6:1221-1231,1990).
In the Nef coding region , SIV mac 239 has an in-frame stop codon after 92aa

15   predicting a truncated product of only 10kD. The remainder of the Nef reading frame
is open and would be predicted to encode a protein of 263aa (30kD) in its fully open
form.

Our starting material for SIVmac239 *nef* gene was a DNA fragment corresponding to

20   the complete coding sequence, cloned on the LX5N plasmid (received from Dr R.C.
Desrosiers, Southborough,MA,USA) .
This SIV *nef* gene is mutated at the premature stop codon (nucleotide G at position
9353 replaces the original T nucleotide) in order to express the full-length SIVmac239
Nef protein.

25

To express this SIV *nef* gene in *Pichia pastoris*, the PHIL-D2-MOD
Vector (previously used for the expression of HIV-1 *nef* and *tat* sequences) was used.
The recombinant protein is expressed under the control of the inducible alcohol
oxidase (AOX1) promoter and the c-terminus of the protein is elongated by a

30   Histidine affinity tail that will facilitate the purification.

10.1 CONSTRUCTION OF THE INTEGRATIVE VECTOR pRIT 14908

35

To construct **pRIT 14908** , the SIV *nef* gene was amplified by PCR from the
pLX5N/SIV-NEF plasmid with primers SNEF1 and SNEF2.

5

PRIMER SNEF1: 5' ATCGT<u>CCATG.G</u>GTGGAGCTATTTT 3'

NcoI

(Seq ID No: 6)

10

PRIMER SNEF2: 5' CGGCTA<u>CTAGT</u>GCGAGTTTCCTT 3'

SpeI

(Seq ID No: 7)

15   The SIV *nef* DNA region amplified starts at nucleotide 9077 and terminates at

nucleotide 9865 (Aids Research and Human Retroviruses, 6:1221-1231,1990).

An NcoI restriction site (with carries the ATG codon of the *nef* gene) was introduced

at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end.

20   The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both

restricted by NcoI and SpeI. Since one NcoI restriction site is present on the SIV *nef*

amplified sequence (at position 9286), two fragments of respectively ±200bp and

± 600bp were obtained, purified on agarose gel and ligated to PHIL-D2-MOD vector.

The resulting recombinant plasmid received, after verification of the *nef* amplified

25   region by automated sequencing, the pRIT 14908 denomination.

10.2 <u>TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115(his4).</u>

30   To obtain *Pichia pastoris* strain expressing SIV *nef*-His, strain GS115 was

transformed with a linear NotI fragment carrying only the expression cassette and the

HIS4 gene (Fig.11).

This linear NotI DNA fragment ,with homologies at both ends with AOX1 resident

P.pastoris gene, favors recombination at the AOX1 locus.

35   Multicopy integrant clones were selected by quantitative dot blot analysis .

One transformant showing the best production level for the recombinant protein was

selected and received the **Y1772** denomination.

5

Strain **Y1772** produces the recombinant **SIV Nef-His** protein, a **272 amino acids** protein which would be composed of:

°Myristic acid

°A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD vector .

10   °262 amino acids (aa) of Nef protein (starting at aa 2 and extending to aa 263, see Figure 12)

°A threonine and a serine created by the cloning procedure (cloning at SpeI site of PHIL-D2-MOD vector (Fig.11 – includes Seq ID No: 29).

°One glycine and six histidines.

15

Nucleic and Protein sequences are shown in Figure 12 (Seq ID Nos: 30 and 31).

10.3 CHARACTERIZATION OF THE EXPRESSED PRODUCT OF STRAIN

20   ## Y1772.

Expression level

After 16 hours induction in medium containing 1% methanol as carbon source,

25   abundance of the recombinant Nef-His protein, was estimated at **10%** of total protein (Fig.13, lanes 3-4).

Solubility

30   Induced cultures of recombinant strain Y1772 producing the Nef-His protein were centrifuged. Cell pellets were resuspended in breaking buffer, disrupted with 0.5mm glass beads and the cell extracts were centrifuged. The proteins contained in the insoluble pellet (P) and in the soluble supernatant (S) were compared on a Coomassie Blue stained SDS-PAGE10%.

35   As shown in figure 13, the majority of the recombinant protein from strain Y1772 (lanes 3-4) is associated with the insoluble fraction.

5   Strain Y1772 which presents a satisfactory recombinant protein expression level is
        used for the production and purification of SIV Nef-His protein.

Example 11: EXPRESSION OF GP120 IN CHO

10

        A stable CHO-K1 cell line which produces a recombinant gP120 glycoprotein has

        been established. Recombinant gP120 glycoprotein is a recombinant truncated form of

        the gP120 envelope protein of HIV-1 isolate W61D. The protein is excreted into the

        cell culture medium, from which it is subsequently purified.

15

        Construction of gp120 transfection plasmid pRIT13968

        The envelope DNA coding sequence (including the 5`exon of tat and rev) of HIV-1

        isolate W61D was obtained (Dr. Tersmette, CCB, Amsterdam) as a genomic gp160

        envelope containing plasmid W61D (Nco-XhoI). The plasmid was designated

20   pRIT13965.

        In order to construct a gp120 expression cassette a stop codon had to be inserted at the

        amino acid glu 515 codon of the gp160 encoding sequence in pRIT13965 using a

        primer oligonucleotide sequence (DIR 131) and PCR technology. Primer DIR 131

25   contains three stop codons (in all open reading frames) and a SalI restriction site.

        The complete gp120 envelope sequence was then reconstituted from the N-terminal

        BamH1-DraI fragment (170 bp) of a gp160 plasmid subclone pW61d env

        (pRIT13966) derived from pRIT13965, and the DraI-SalI fragment (510 bp) generated

30   by PCR from pRIT13965. Both fragments were gel purified and ligated together into

        the E.coli plasmid pUC18, cut first by SalI (klenow treated), and then by BamH1. This

        resulted in plasmid pRIT13967. The gene sequence of the XmaI-SalI fragment (1580

        bp) containing the gp120 coding cassette was sequenced and found to be identical to

        the predicted sequence. Plasmid RIT13967 was ligated into the CHO GS-expression

35   vector pEE14 (Celltech Ltd., UK) by cutting first with BclI (klenow treated) and then

        by XmaI. The resulting plasmid was designated pRIT13968.

Preparation of Master Cell Bank

The gp120-construct (pRIT13968) was transfected into CHO cells by the classical CaPO$_4$-precipitation/glycerol shock procedure. Two days later the CHOK1 cells were subjected to selective growth medium (GMEM + methionine sulfoximine (MSX) 25 µM + Glutamate + asparagine + 10% Foetal calf serum). Three chosen transfectant clones were further amplified in 175m^2 flasks and few cell vials were stored at -80°C. C-env 23,9 was selected for further expansion.

A small prebank of cells was prepared and 20 ampoules were frozen. For preparation of the prebank and the MCB, cells were grown in GMEM culture medium, supplemented with 7.5 % fetal calf serum and containing 50 µM MSX. These cell cultures were tested for sterility and mycoplasma and proved to be negative.

The Master Cell Bank CHOK1 env 23.9 (at passage 12) was prepared using cells derived from the premaster cell bank. Briefly, two ampoules of the premaster seed were seeded in medium supplemented with 7.5% dialysed foetal bovine serum. The cells were distributed in four culture flasks and cultured at 37°C. After cell attachment the culture medium was changed with fresh medium supplemented with 50 µM MSX. At confluence, cells were collected by trypsination and subcultured with a 1/8 split ratio in T-flasks - roller bottle - cell factory units. Cells were collected from cell factory units by trypsination and centrifugation. The cell pellet was resuspended in culture medium supplemented with DMSO as cryogenic preservative. Ampoules were prelabelled, autoclaved and heat-sealed (250 vials). They were checked for leaks and stored overnight at -70°C before storage in liquid nitrogen.

Cell Culture And Production Of Crude Harvest

Two vials from a master cell bank are thawed rapidly. Cells are pooled and inoculated in two T-flasks at 37° ± 1°C with an appropriate culture medium supplemented with 7.5 % dialysed foetal bovine (FBS) serum. When reaching confluence (passage 13), cells are collected by trypsinisation, pooled and expanded in 10 T-flasks as above. Confluent cells (passage 14) are trypsinised and expanded serially in 2 cell factory units (each 6000 cm^2; passage 15), then in 10 cell factories (passage 16). The growth culture medium is supplemented with 7.5 % dialysed foetal bovine (FBS) serum and

5   1% MSX. When cells reach confluence, the growth culture medium is discarded and

replaced by "production medium" containing only 1 % dialysed foetal bovine serum

and no MSX. Supernatant is collected every two days (48 hrs-interval) for up to 32

days. The harvested culture fluids are clarified immediately through a 1.2-0.22 μm

filter unit and kept at -20°C before purification.

10

**Example 12: PURIFICATION OF HIV GP 120 (W61D CHO) FROM CELL
CULTURE FLUID**

All purifiaction steps are performed in a cold room at 2-8°C. pH of buffers are

15   adjusted at this temperature and are filtered on 0.2 μm filter. They are tested for

pyrogen content (LAL assay). Optical density at 280 nm, pH and conductivity of

column eluates are continuously monitored.

(i)   Clarified Culture Fluid

20   The harvested clarified cell culture fluid (CCF) is filter-sterilized and Tris buffer, pH

8.0 is added to 30 mM final concentration. CCF is stored frozen at -20°C until

purification.

(ii)   Hydrophobic Interaction Chromatography

25   After thawing, ammonium sulphate is added to the clarified culture fluid up to 1 M.

The solution is passed overnight on a TSK/TOYOPEARL-BUTYL 650 M

(TOSOHAAS) column, equilibrated in 30 mM Tris buffer- pH 8.0 - 1 M ammonium

sulphate. Under these conditions, the antigen binds to the gel matrix. The column is

washed with a decreasing stepwise ammonium sulphate gradient. The antigen is

30   eluted at 30 mM Tris buffer- pH 8.0 - 0.25 M ammonium sulphate.

(iii)   Anion-exchange Chromatography

After reducing the conductivity of the solution between 5 and 6 mS/cm, the gP120

pool of fractions is loaded onto a Q-sepharose Fast Flow (Pharmacia) column,

35   equilibrated in Tris-saline buffer - pH 8.0. The column is operated on a negative

mode, i.e. gP120 does not bind to the gel, while most of the impurities are retained.

5   (iv)   Concentration and diafiltration by ultrafiltration

In order to increase the protein concentration, the gP120 pool is loaded on a

FILTRON membrane "Omega Screen Channel", with a 50 kDa cut-off. At the end of

the concentration, the buffer is exchanged by diafiltration with 5 mM phosphate buffer

containing $CaCl_2$ 0.3 mM, pH 7.0. If further processing is not performed

10   immediately, the gP120 pool is stored frozen at -20°C. After thawing the solution is

filtered onto a 0.2 μM membrane in order to remove insoluble materiel.

(v)   Chromatography on hydroxyapatite

The gP120 UF pool is loaded onto a macro-Prep Ceramic Hydroxyapatite, type II

15   (Biorad) column equilibrated in 5 mM phosphate buffer + $CaCl_2$ 0.3 mM, pH 7.0.

The column is washed with the same buffer. The antigen passes through the column

and impurities bind to the column.

(vi)   Cation exchange chromatography

20   The gP120 pool is loaded on a CM/TOYOPEARL-650 S (TOSOHAAS) column

equilibrated in acetate buffer 20 mM, pH 5.0. The column is washed with the same

buffer, then acetate 20 mM, pH 5.0 and NaCl 10 mM. The antigen is then eluted by

the same buffer containing 80 mM NaCl.

25   (vii)   Ultrafiltration

In order to augment the virus clearance capacity of the purification process, an

additional ultrafiltration step is carried out. The gP120 pool is subjected to

ultrafiltration onto a FILTRON membrane "Omega Screen Channel", cut-off 150 kDa.

This pore-size membrane does not retain the antigen. After the process, the diluted

30   antigen is concentrated on the same type of membrane (Filtron) but with a cut-off of

50 kDa.

(viii)   Size exclusion Gel Chromatography

The gP120 pool is applied to a SUPERDEX 200 (PHARMACIA) column in order to

35   exchange the buffer and to eliminate residual contaminants. The column is eluted

with phosphate buffer saline (PBS).

5

(ix)   <u>Sterile filtration and storage</u>

Fractions are sterilized by filtration on a 0.2 µM PVDF membrane (Millipore). After sterile filtration, the purified bulk is stored frozen at -20°C up to formulation. The purification scheme is summarized by the flow sheet below.

10

⇒ Level of purity of the purified bulk estimated by SDS-PAGE analysis (Silver staining / Coomassie Blue / Western Blotting) is ≥ 95%.

⇒ Production yield is around 2.5 mg /L CCF (according to Lowry assay) - Global purification yield is around 25% (according to Elisa assay)

15   ⇒ Purified material is stable 1 week at 37°C (according to WB analysis)

Purification of gp120 from culture fluid

20   Mark √ indicate steps that are critical for virus removal.

CLARIFIED CULTURE FLUID
↓

HYDROPHOBIC INTERACTION CHROMATOGRAPHY
(BUTYL -TOYOPEARL 650 M)
↓

ANION EXCHANGE CHROMATOGRAPHY (NEGATIVE   √
MODE)
(Q-SEPHAROSE)
↓

50 KD ULTRAFILTRATION
(CONCENTRATION AND BUFFER EXCHANGE)
↓

(STORAGE -20°C)

↓

HYDROXYAPATITE CHROMATOGRAPHY (NEGATIVE
MODE)
(MACROPREP CERAMIC HYDROXYAPATITE II)
↓

CATION EXCHANGE CHROMATOGRAPHY
(CM-TOYOPEARL 650 S)
↓

150 KD ULTRAFILTRATION   √
(OMEGA MEMBRANES / FILTRON)
↓

50 KD ULTRAFILTRATION
(CONCENTRATION)
↓

SIZE EXCLUSION CHROMATOGRAPHY   √
(SUPERDEX 200)
STERILE FILTRATION
↓

PURIFIED BULK
STORAGE -20°C

5

5　　**Example 13: VACCINE PREPARATION**

A vaccine prepared in accordance with the invention comprises the expression products of one or more DNA recombinants encoding an antigen. Furthermore, the formulations comprise a mixture of 3 de -O-acylated monophosphoryl lipid A 3D-

10　MPL and QS21 in an oil/water emulsion or an oligonucleotide containing unmethylated CpG dinucleotide motifs and aluminium hydroxide as carrier.

3D-MPL: is a chemically detoxified form of the lipopolysaccharide (LPS) of the Gram-negative bacteria Salmonella minnesota.

15

Experiments have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral immunity and a T_{H1} type of cellular immunity.

QS21: is a saponin purified from a crude extract of the bark of the Quillaja Saponaria

20　Molina tree, which has a strong adjuvant activity: it induces both antigen-specific lymphoproliferation and CTLs to several antigens.
Experiments have demonstrated a clear synergistic effect of combinations of 3D-MPL and QS21 in the induction of both humoral and T_{H1} type cellular immune responses.

25　**The oil/water emulsion** is composed of 2 oils (a tocopherol and squalene), and of PBS containing Tween 80 as emulsifier. The emulsion comprises 5% squalene, 5% tocopherol, 2% Tween 80 and has an average particle size of 180 nm (see WO 95/17210).

30　Experiments performed have proven that the adjunction of this O/W emulsion to 3D-MPL/QS21 further increases their immunostimulant properties.

Preparation of the oil/water emulsion (2 fold concentrate)

35　Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is

5   added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S Microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

Preparation of oil in water formulation.

10   Antigens (100 μg gp120, 20 μg NefTat, and 20 μg SIV Nef, alone or in combination) were diluted in 10 fold concentrated PBS pH 6.8 and H_2O before consecutive addition of the oil in water emulsion, 3D-MPL (50μg), QS21 (50μg) and 1 μg/ml thiomersal as preservative at 5 min interval. The emulsion volume is equal to 50% of the total volume (250μl for a dose of 500μl).

15

       All incubations were carried out at room temperature with agitation.

       CpG oligonucleotide (CpG) is a synthetic unmethylated oligonucleotide containing one or several CpG sequence motifs. CpG is a very potent inducer of T_{H1} type
20   immunity compared to the oil in water formulation that induces mainly a mixed T_{H1}/T_{H2} response. CpG induces lower level of antibodies than the oil in water formulation and a good cell mediated immune response. CpG is expected to induce lower local reactogenicity.

25   Preparation of CpG oligonucleotide solution: CpG dry powder is dissolved in H_2O to give a solution of 5 mg/ml CpG.

Preparation of CpG formulation.

       The 3 antigens were dialyzed against NaCl 150 mM to eliminate the phosphate ions
30   that inhibit the adsorption of gp120 on aluminium hydroxide.
       The antigens diluted in H_2O (100 μg gp120, 20 μg NefTat and 20 μg SIV Nef) were incubated with the CpG solution (500 μg CpG) for 30 min before adsorption on $Al(OH)_3$ to favor a potential interaction between the His tail of NefTat and Nef antigens and the oligonucleotide (stronger immunostimulatory effect of CpG
35   described when bound to the antigen compared to free CpG). Then were consecutively added at 5 min interval $Al(OH)_3$ (500 μg), 10 fold concentrated NaCl and 1 μg/ml thiomersal as preservative.

5

All incubations were carried out at room temperature with agitation.

Example 14: IMMUNIZATION AND SHIV CHALLENGE EXPERIMENT IN

10 **RHESUS MONKEYS.**

First Study

Groups of 4 rhesus monkeys were immunized intramuscularly at 0, 1 and 3 months

15 with the following vaccine compositions:

Group 1:	Adjuvant 2	+ gp120		
Group 2:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 3:	Adjuvant 2		+ NefTat*	+ SIV Nef
Group 4	Adjuvant 6	+ gp120	+ NefTat	+ SIV Nef
Group 5	Adjuvant 2		+ NefTat	+ SIV Nef
Group 6	Adjuvant 2			

Adjuvant 2 comprises squalene/tocopherol/Tween 80/3D-MPL/QS21 and

25 Adjuvant 6 comprises alum and CpG.

Tat* represents mutated Tat, in which Lys41→Ala and in RGD motif Arg78→Lys

and Asp80→Glu (Virology 235: 48-64, 1997).

30 One month after the last immunization all animals were challenged with a pathogenic

SHIV (strain 89.6p). From the week of challenge (wk16) blood samples were taken

periodically at the indicated time points to determine the % of CD4-positive cells

among peripheral blood mononuclear cells by FACS analysis (Figure 14) and the

concentration of RNA viral genomes in the plasma by bDNA assay (Figure 15).

35

Results

5   All animals become infected after challenge with SHIV$_{89.6p}$.

CD4-positive cells decline after challenge in all animals of groups 1, 3, 5 and 6 except one animal in each of groups 1 and 6 (control group). All animals in group 2 exhibit a slight decrease in CD4-positive cells and recover to baseline levels over time. A

10   similartrend is observed in group 4 animals (Figure 14).

Virus load data are almost the inverse of CD4 data. Virus load declines below the level of detection in ¾ group 2 animals (and in the one control animal that maintains its CD4-positive cells), and the fourth animal shows only marginal virus load. Most

15   of the other animals maintain a high or intermediate virus load (Figure 15).

Surprisingly, anti-Tat and anti-Nef antibody titres measured by ELISA were 2 to 3-fold higher in Group 3 (with mutated Tat) than in Group 5 (the equivalent Group with non-mutated Tat) throughout the course of the study.

20

At week 68 (56 weeks post challenge) all animals from the groups that had received the full antigen combination (groups 2 and 4) were still alive, while most of the animals in the other groupshad to be euthanized due to AIDS-like symptoms. The surviving animals per group were:

25

        Group 1:   2/4
        Group 2:   4/4
        Group 3:   0/4
        Group 4   4/4
30   Group 5   0/4
        Group 6   1/4

Conclusions

35

The combination of gp120 and NefTat (in the presence of SIV Nef) prevents the loss of CD4-positive cells, reduces the virus load in animals infected with pathogenic

5   SHIV$_{89.6p}$, and delays or prevents the development of AIDS-like disease symptoms, while gp120 or NefTat/SIV Nef alone do not protect from the pathologic consequences of the SHIV challenge.

The adjuvant 2 which is an oil in water emulsion comprising squalene, tocopherol and

10   Tween 80, together with 3D-MPL and QS21 seems to have a stronger effect on the study endpoints than the alum / CpG adjuvant.

Second study

15   A second rhesus monkey SHIV challenge study was conducted to confirm the efficacy of the candidate vaccine gp120/NefTat + adjuvant and to compare different Tat-based antigens. The study was conducted by a different laboratory.

The design of the study was as follows.

20

Groups of 6 rhesus monkeys were immunized at 0, 4 and 12 weeks with injections i.m. and challenged at week 16 with a standard dose of pathogenic SHIV$_{89.6p.}$

Group 1 is the repeat of Group 2 in the first study.

25

Group 1:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 2:	Adjuvant 2	+ gp120	+ Tat (oxidised)	
Group 3:	Adjuvant 2	+ gp120	+ Tat (reduced)	
Group 4	Adjuvant 2			

30

The follow-up/endpoints were again % CD4-positive cells, virus load by RT-PCR, morbidity and mortality

Results

35

All animals except one in group 2 become infected after challenge with SHIV$_{89.6p.}$

5   CD4-positive cells decline significantly after challenge in all animals of control group
       4 and group 3, and in all but one animals of group 2. Only one animal in group 1
       shows a marked decrease in CD4-positive cells. Unlike the animals from the first
       study, the monkeys in the second experiment display a stabilisation of CD4-positive
       cells at different levels one month after virus challenge (Figure 16). The stabilisation
10   is generally lower than the initial % of CD4-positive cells, but will never lead to a
       complete loss of the cells. This may be indicative of a lower susceptibility to SHIV-
       induced disease in the monkey population that was used for the second study.
       Nonetheless, a beneficial effect of the gp120/NefTat/SIV Nef vaccine and the two
       gp120/Tat vaccines is demonstrable. The number of animals with a % of CD4-positive
15   cells above 20 is 5 for the vaccinated animals, while none of the control animals from
       the adjuvant group remains above that level.

       Analysis of RNA plasma virus loads confirms the relatively low susceptibility of the
       study animals (Figure 17). Only 2 of the 6 control animals maintain a high virus load,
20   while the virus disappears from the plasma in the other animals. Thus, a vaccine effect
       is difficult to demonstrate for the virus load parameter.

       Conclusions

25   Analysis of CD4-positive cells indicates that the vaccine gp120/NefTat + adjuvant (in
       the presence of SIV Nef) prevents the drop of CD4-positive cells in most vaccinated
       animals This is a confirmation of the result obtained in the first SHIV study. Due to
       the lack of susceptibility of the study animals, the virus load parameter could not be
       used to demonstrate a vaccine effect. Taken together, the combination of gp120 and
30   Tat and Nef HIV antigens provides protection against the pathologic consequences of
       HIV infection, as evidenced in a SHIV model.

       The Tat alone antigens in combination with gp120 also provide some protection from
       the decline of CD4-positive cells. The effect is less pronounced than with the
35   gp120/NefTat/SIV Nef antigen combination, but it demonstrates that gp120 and Tat
       are able to mediate some protective efficacy against SHIV-induced disease
       manifestations.

59

5   The second SHIV challenge study was performed with rhesus monkeys from a source
         completely unrelated to the source of animals from the first study. Both parameters, %
         of CD4-positive cells and plasma virus load, suggest that the animals in the second
         study were less susceptible to SHIV-induced disease, and that there was considerably
10   greater variability among the animals. Nonetheless, a beneficial effect on the
         maintenance of CD4-positive cells of the gp120/NefTat/SIV Nef vaccine was seen
         with the experimental vaccine containing gp120/NefTat and SIV Nef. This indicates
         that the vaccine effect was not only repeated in a separate study, but furthermore
         demonstrated in an unrelated monkey population.

15

Example 15:   Generation of PMID vectors for gp120 and Nef/Tat

         Expression vectors were constructed as described below. Endotoxin free plasmid
         preps were prepared and used to transfect subconfluent 293T cell monolayers in 24
20   well tissue culture plates with 1μg of DNA using Lipofectamine 2000. Samples were
         harvested 24 hours post transfection and examined by Western Blot to assess
         expression levels. (Fig 18 and 19)

         Codon optimisation of gp120 resulted in a substantial increase of REV independent
25   expression.

Generation of plasmids

         See also Figures 20 – 23

30

gp120:
         Both wild type and optimised gp120 were compared.

Wild Type: (pgp120w)
35   PCR cloned from pRIT 13968 (see Example 11) using primers:
         g120w-5'   GAATTCGCGGCCGCAATGAAAGTGAAGGAGACCAG
         (Seq ID No: 32)

5   g120w-3'   GAATTCGGATCCTTATCTCTGCACCACTCTTC

(Seq ID No: 33)

The gene was cloned into vector p7313-ie as a NotI-BamHI fragment and sequenced.

A single base change (conservative) was found relative to the reference sequence, but

10   this change was also found on sequencing pRIT 13968 (T1170C in gp120).

Codon Optimised (pgp120c)

The gene sequence was based on the gp120 sequence from pRIT 13968. This has a

RSCU value of 0.297. Optimisation was performed using SynGene 2d, resulting in a

15   RSCU value of 0.749. The sequence was split into 40 overlapping oligonucleotide,

PCR assembled and recovered using the end primers. The gene was cloned into vector

p7313-ie as a NotI-BamHI fragment and sequenced. Restriction fragments from three

initial clones were combined to generate a single correct clone.

20   **Nef/Tat** (pNTm and ptrNTm)

The gene for the Nef/Tat fusion protein was provided in plasmid pRIT15244. The

plasmid pRIT 15244 is identical to pRIT 14913 previously described except that the

His tail has been deleted. The Tat in this plasmid contains three mutations as

previously described. The fusion contains full length Nef which has an immune

25   modulatory function (Collins and Baltimore (1999)) that may be abrogated by N-

terminal truncation. Therefore constructs were generated for both full length

Nef/mutant Tat(pNTm) and truncated Nef/mutant Tat(ptrNTm), in which the first 65

amino acids of Nef were removed. These sequences were PCR amplified from

pRIT15244 using primers:

30

5'Nef: GAATTCGCGGCCGCCATGGGTGGCAAGTGGTCAAAAAG

5'trNef: GAATTCGCGGCCGCCATGGTGGGTTTTCCAGTCACACC

3'Tat: GAATTCGGATCCTTATTCCTTCGGGCCTGTCGGG

(Seq ID Nos: 34, 35 and 36 respectively)

35   The genes were cloned into vector p7313-ie as NotI-BamHI fragments and sequenced.

Dual expression vectors: (pRIX1 and pRIX2)

5

The Nef/Tat and trNef/Tat expression cassettes were excised as ClaI-XmnI restriction fragments, and ligated into the ClaI and blunted Sse8387 I sites of the vector containing the codon optimised gp120 to provide single plasmids for expression of both proteins (pRIX1 and pRIX2 respectively).

10

Composition of plasmid p7313-ie

The plasmid was constructed by replacing the beta-lactamase gene containing Eam1105I - Pst1 fragment of pUC19 (available from Amersham Pharmacia Biotech

15 UK Ltd., Amersham Place, Little Chalfont, Bucks, HP7 9NA) with an EcoRI fragment of pUC4K (Amersham-Pharmacia) containing the Kanamycin resistance gene, following blunt ending of both fragments using T4 DNA polymerase. The human Cytomegalovirus IE1 promoter /enhancer, Intron A, was derived from plasmid JW4303 obtained from Dr Harriet Robinson, University of Massachusetts, and

20 inserted into the Sal1 site of pUC19 as a XhoI -Sal1 fragment, incorporating the bovine growth hormone polyadenylation signal. Deletion of the 5' SalI-BanI fragment from the promoter generated the minimal promoter used in the vector (WO00/23592 - Powderject Vaccines Inc.). HBV Surface antigen 3'UTR was derived from Hepatitis B Virus, serotype adw, in the vector pAM6 (Moriarty et al., Proc.Natl.Acad.Sci. USA,

25 78, 2606-10, 1981). pAM6 (pBR322 based vector) was obtained from the American Type Culture Collection, catalogue number ATCC 45020. The 3'UTR was inserted 5' to the polyadenylation signal as a 1.4kb BamHI fragment, blunt ended for insertion to remove the BamHI sites. In a series of steps (including digestion with *Bgl* II, Klenow polymerase treatment, digestion with *BstX* I, digestion with *Nco* I, treatment with

30 mung bean nuclease to remove overhang and further digestion with *BstX* I), modifications were made to the region between the 3'untranslated enhancer region of the HBV S gene and bGHpA signal to remove all open reading frames of greater than 5 codons between the X gene promoter and the bGHpA signal. This resulted in deletion of sequence encoding the translatable portion of the X protein (9 amino acids)

35 and the X gene start codon. The bovine growth hormone polyadenylation signal was substituted with the rabbit beta globin polyadenylation signal. The 5'non-coding and

5   coding sequences of the S antigen were excised and replaced with an oligonucleotide

linker to provide multiple cloning sites as shown to produce plasmid p7313-PL.

Hind---NotI--   EcoRV-   -NdeI-   -BamHI

10   AGCTTGCGGCCGCTAGCGATATCGGTACCATATGTCGACGGATCC....

     ....ACGCCGGCGATCGCTATAGCCATGGTCTACAGCTGCCTAGGCCGG

          -NheI-   -KpnI-   -SalI-   ΔNotI

(Seq ID No: 37)

15   This polylinker was further extended by insertion of an additional oligonucleotide

linker between the KpnI and SalI sites:

AspI-   -MunI- NaeI-   NdeI--   BglII-

GTACCGGTCAATTGGCGCCGGCGCGCCATATGACGTCAGATCTG----

20   ----GCCAGTTAACCGCGGCCGCGCGGTATACTGCAGTCTAGACAGCT

--AgeI-   -NarI--   AatII-   SalI

(Seq ID No: 38)

The ColE1 cer sequence was obtained from a subclone from plasmid pDAH212 from

25   David Hodgeson (Warwick University) and amplified by PCR using primers to place

EcoRI restriction sites at the ends of the sequence. The cer sequence was then inserted

into the EcoRI site of p7313-PL to produce plasmid p7313-PLc. The sequence of the

amplified cer was verified against the Genbank entry M11411.

The HBV 3'UTR sequence between the promoter and polyadenylation signal was

30   removed by PCR amplification of the polyadenylation signal using primers:

sense: CCATGGATCCGATCTTTTTCCCTCTGCC (Seq ID No: 39)

antisense: GTTAGGGTGAAAAGCTTCCGAGTGAGAGACAC (Seq ID No: 40)

The resulting product was cut with BamHI and XmnI and used to replace the

corresponding fragment containing both the polyadenylation signal and the 3'UTR.

35   The Intron A sequence was removed from the plasmid by PCR amplification of the

CMV promoter/enhancer using primers:

sense: GCTAGCCTGCAGGCTGACCGCCCAACGAC (Seq ID No: 41)

5   antisense: GTTCTCCATCGCGGCCGCACTCTTGGCACGGGG (Seq ID No: 42)

The resulting product was cut with Sse8387 I and NotI, and inserted back into the

Sse8387 I and NotI sites of the parental vector.

10 **Example 16: IMMUNOGENICITY STUDIES**

A mouse immunogenicity study may be performed to test the immunogenicity of

gp120 and NefTat constructs delivered by particle mediated immunotherapeutic

delivery (PMID) to show that the construct can generate an immune response *in vivo*.

15 The DNA constructs may be used in combination with protein to determine whether

there is an advantage of the combined approach compared to immunisation with the

individual components. The aim is to detect and quantify cellular and humoral

immune responses in mice following priming with protein and boosting with DNA

(PMID) or priming with DNA (PMID) and boosting with protein.

20

The DNA is precipitated onto gold microparticles, which are used to coat the inner

walls of a tefzel cartridge. PMID cartridges are prepared using a DNA loading rate of

2, which gives approximately 0.5 µg DNA per cartridge.

25 Mice are immunised by PMID on the abdomen by providing two shots containing

gp120 or NetTat. Following a PMID boost spleens are removed and the spleen cells

are used to determine immunogenicity to individual constructs. Responses are

evaluated using the IFN-γ ELIspot assay. In this assay peptides from gp120 and Nef

are used to stimulate the spleen cells to secrete IFNγ which is captured by an antibody

30 based detection system. The measurement of humoral responses to gp120 and Nef are

measured by ELISA.

gp120/NefTat Prime Boost Studies

35 The aim of the study is to detect and quantify cellular and humoral immune responses

in mice following priming with protein and boosting with DNA (PMID) or priming

with DNA (PMID) and boosting with protein.

64

5

Cartridge Preparation

Gene gun cartridges are suitably prepared using a DNA loading rate (DLR) of 2, which will give approximately 0.5 µg DNA per cartridge.

10

In vivo Immunogenicity

An experiment (Experiment I) may suitably be carried out as set out in the table below. The readouts for this experiment are carried out by IFN-γ ELIspot to Balb/c

15 peptides from gp120 and Nef to measure the cellular responses and detection of antibodies to gp120 and Nef by ELISA for the humoral response.

Experiment I

Group	Mouse strain	Prime	Boost
A	Balb/c Female	PMID: gp120, 2 x 0.5 µg	PMID: gp120, 2 x 0.5 µg
B	Balb/c Female	PMID: NefTat, 2 x 0.5 µg	PMID: NefTat, 2 x 0.5 µg
C	Balb/c Female	PMID: Empty vector, 2 x 0.5 µg	PMID: Empty vector, 2 x 0.5 µg
D	Balb/c Female	Protein: gp120	Protein: gp120
E	Balb/c Female	Protein: NefTat	Protein: NefTat
H	Balb/c Female	Protein: Irrelevant protein	Protein: Irrelevant protein

n = 3

20

A further experiment (Experiment II) may be carried out as follows:

Experiment II

Group	Mouse strain	Prime	Boost
A	Balb/c Female	PMID: gp120, 2 x 0.5 µg	Protein: gp120
B	Balb/c	PMID: gp120, 2 x 0.5 µg	PMID: gp120, 2 x 0.5 µg

	Female		
C	Balb/c Female	PMID: NefTat, 2 x 0.5 µg	Protein: NefTat
D	Balb/c Female	PMID: NefTat, 2 x 0.5 µg	PMID: NefTat, 2 x 0.5 µg
L	Balb/c Female	PMID: gp120, 2 x 0.5 µg NefTat, 2 x 0.5 µg	Protein: gp120 + NefTat
L	Balb/c Female	PMID: gp120, 2 x 0.5 µg NefTat, 2 x 0.5 µg	PMID: gp120, 2 x 0.5 µg NefTat, 2 x 0.5 µg
G	Balb/c Female	PMID: Empty vector, 2 x 0.5 µg	Protein: Irrelevant protein
H	Balb/c Female	Protein: gp120	PMID: gp120, 2 x 0.5 µg
I	Balb/c Female	Protein: gp120	Protein: gp120
I	Balb/c Female	Protein: NefTat	PMID: NefTat, 2 x 0.5 µg
K	Balb/c Female	Protein: NefTat	Protein: NefTat
L	Balb/c Female	Protein: gp120 + NefTat	PMID: gp120, 2 x 0.5 µg NefTat, 2 x 0.5 µg
M	Balb/c Female	Protein: gp120 + NefTat	Protein: gp120 + NefTat
N	Balb/c Female	Protein: Irrelevant protein	PMID: Empty vector, 2 x 0.5 µg

5   n = 3

Sample protocol

Female Balb/c mice are used. All mice are pre-bled (tail vein) before the start of the

10   experiment.

Groups of 3 mice/group are culled after the primary immunisation and spleens are

collected for enumeration of IFN-γ -producing CD8 cells by ELIspot assay using

gp120 and Nef K^d-restricted peptides.

15   Groups of 3 mice/group are culled at a minimum of two time points after the

secondary immunisation and cardiac blood samples are collected. These are analysed

for antibodies to gp120 and Nef using ELISA assays. Spleens are also be collected for

enumeration of IFN-γ -producing CD8 cells by ELIspot assay using gp120 and Nef

K^d-restricted peptides.

5

Immunological Assays

ELIspot assays

Cellular immune responses are detected using Interferon-γ ELIspot assays.

Splenocytes will be isolated from 3 mice per group at each selected time point (one

10   post primary and a minimum of two post-boost), and incubated overnight with

peptides to known CD8 epitopes (restricted to a K^d (Balb/c) background) in plates

coated with α--Interferon-γ. The splenocytes are lysed and plates developed using a

secondary α--Interferon-γ antibody and biotin-streptavidin amplification system.

15   ELISA assays

Humoral responses are detected using standard antibody ELISA assays. 96-well flat-

bottomed microtitre plates will be coated with protein and blocked. Serial dilutions of

serum collected prior to immunisation and after boost are collected and incubated in

the plates. The plates are developed after incubation with an anti-mouse antibody.

20   Responses to gp120, Nef and Tat are analysed.

Example 17: IMMUNOGENICITY STUDIES

25   **Protocol**

Cartridges were prepared for particle mediated delivery using a gene gun using

standard methods. A DNA loading rate of 2 which will give approximately 0.5 μg

DNA/cartridge was used.

30   F1 (C3H x Balb/c) mice were given a primary immunisation with either gp120 protein

and NefTat in adjuvant (administered via the intramuscular route) or with gp120

DNA, codon optimised and cloned into vector p7313-ie as described in Example 15,

and a vector expressing a Nef fusion protein (using particle mediated delivery wherein

the DNA is coated onto gold beads). The mice were boosted 23 days later either with

35   protein in adjuvant (administered via the intramuscular route) or with DNA (using

particle mediated delivery wherein the DNA is coated onto gold beads). Mice were

culled 5 days later and spleens were collected. The splenocytes were harvested by

5   teasing out, erythocytes were lysed and splenocytes were washed and counted.
       Specialised ELIspot plates (coated with interferon-gamma capture antibody and
       blocked) were used. Splenocytes were transferred to these plates and incubated
       overnight at 37°C/5% CO_2 in the presence of medium control gp120 E7 peptide or
       various nef peptides. The splenocytes were lysed and the plate developed using

10   standard procedures to demonstrate the number of interferon-gamma secreting cells
       present.

       Conclusion

       Although the ELIspot assay was carried out using a sub-optimal concentration of

15   gp120 E7 peptide, the results indicated that the most effective schedule was to prime
       mice with protein in adjuvant (administered i.m.) and then boost with DNA
       (administered by particle mediated delivery). See Figures 24. Similar results were
       obtained for nef, with the two peptides Nef 19 and 20 only being recognised by mice
       that had been primed with protein and boosted with DNA. The sequences for these

20   two peptides are Nef 29: HIV-1 Bru (171-190) GMDDPEREVLEWRFDSRLAF (Seq
       ID No: 43) and Nef 20: HIV-1 Bru (181-200) EWRFDSRLAFHHVARELHPE (Seq
       ID No: 44). See Figure 25.

       Example 18: IMMUNOGENICITY STUDIES
25
       Protocol

       For PMID immunisations (DNA) cartridges were prepared using standard methods. A
       DNA loading rate of 2, which will give approximately 0.5 μg DNA/cartridge was used
       and each immunisation consisted of two shots. Protein was formulated in adjuvant

30   comprising squalene/tocopherol/Tween 80/3D-MPL/QS21 just before use. Balb/c
       mice were given a primary immunisation of either gp120 protein in adjuvant
       (administered via the intramuscular route) or with gp120 codon optimised DNA
       prepared as described in Example 15 (using PMID). The mice were boosted 21 days
       later with either protein in adjuvant (administered via the intramuscular route) or with

35   DNA (using PMID). Mice were culled 7 days later and spleens were collected. The
       splenocytes were harvested by teasing out the spleen cells and erythrocytes were lysed.
       The splenocytes were washed and counted. Specialised ELIspot plates (coated with

5   interferon-gamma capture antibody and blocked) were used. Splenocytes were transferred to these plates and incubated overnight at $37^{\circ}C/5\%$ CO_2 in the presence of pools of gp120 15-mer peptides. The splenocytes were lysed and the plate developed using standard procedures to demonstrate the number of interferon-gamma secreting cells present. Results are shown in Figure 26.

10

Conclusion

Three pools of gp120 15-mer peptides were recognised by mice that had been primed with protein and boosted with DNA. Responses to these three pools of gp120 15-mers were not detected in animals that had been primed with DNA and boosted with

15   protein or immunised twice with either protein or DNA.

CLAIMS

1.   Use of a) an HIV Tat protein or polynucleotide; or

b) an HIV Nef protein or polynucleotide; or

c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or

polynucleotide;

and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine suitable

for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans

against HIV, wherein the protein or polynucleotide is delivered via a bombardment

approach.

2.   Use according to claim 1 wherein the bombardment approach comprises

propelling particles into a target tissue of interest, typically the skin.

3.   Use according to claim 2 wherein the particles are gold beads onto which the

protein or polynucleotide has been coated.

4.   Use according to claim 2 or claim 3 in which the particles are accelerated to

high speed by a helium gas jet.

5.   Use according to any one of claims 2 to 4 wherein the gold beads are 0.4-4.0

μm in diameter

6.   Use according to claim 5 wherein the gold beads are 0-6 to 2.0 μm in diameter.

7.   Use according to any preceding claim wherein the polynucleotide encoding the

nef, tat or gp120 is codon-optimised DNA.

8.   Use according to any preceding claim in which the polynucleotide encoding

the nef, tat and gp120 is present on a single vector.

9.   Use according to claim 8 in which the vector comprises the nef, tat and gp 120

polynucleotides inserted 3' to an enhanced HCMV IE1 promoter.

10.   Use according to claim 9 in which the vector is p7313.

11.   Use according to any preceding claim in which additional regulatory or

structural proteins of HIV such as Rev, Vif, Vpu, and Vpr or proteins derived from the

HIV *gag* or *pol* genes (and/or polynucleotides encoding such regulatory or structural

proteins) are included in the vaccine formulation.

12.   A recombinant DNA molecule comprising a Nef and/or Tat and/or gp 120

gene in a vector in which the gene of interest is inserted 3' to an enhanced HCMV IE1

promoter.

13.   A recombinant DNA molecule according to claim 12 in which the vector is p 7313.

14.   A recombinant DNA molecule according to claim 12 or claim 13 in which at least one of the genes of interest is codon-optimised.

15.   A recombinant DNA molecule in which the gp 120 DNA is codon-optimised.

16.   Use of a recombinant DNA molecule according to any one of claims 12 to 15 in the manufacture of an HIV vaccine for the prophylactic or therapeutic immunisation of humans.

17.   A plurality of particles, preferably gold particles, coated with recombinant DNA comprising a Nef and/or Tat and/or gp 120 gene in a vector.

18.   Particles according to claim 17 coated with DNA comprising a Nef, a Tat and a gp 120 gene in a single vector, preferably wherein the Nef and Tat gene are in the form of a NefTat fusion.

19.   Particles according to claim 17 or 18 wherein the DNA encoding at least one of the Nef, Tat or gp120 is codon optimised for expression in human cells.

20.   Particles according to any one of claims 17 to 19 wherein one or more of the genes of interest is inserted 3' to an enhanced HCMV IE1 promoter.

21.   Particles according to any one of claims 17 to 20 in which the vector is P7313.

22.   A kit comprising at least two different vaccine compositions including a) a composition comprising a plurality of particles coated with DNA encoding gp120 and nef and/or tat or neftat as described herein and b) a composition comprising gp120 and nef and/or tat or neftat DNA or proteins as described herein wherein the DNA or proteins in b) are not coated onto particles.

FIGURE 1

The DNA and amino acid sequences of Nef-His; Tat-His; Nef-Tat-His fusion and mutated Tat is illustrated.

Pichia-expressed constructs (plain constructs)

⇒ *Nef - HIS*

DNA sequence (Seq. ID. No. 8)

```
ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGG
GGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGC
CACCATCACCATCACCATTAA
```

Protein sequence(Seq. ID. No. 9)

```
MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWI
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLH
GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHHHHHH.
```

⇒ *Tat - HIS*

DNA sequence (Seq. ID. No. 10)

```
ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAA
ACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTC
ATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAA
```

TCCCGAGGGGACCCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCAT
TAA

Protein sequence (Seq. ID. No. 11)

MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRR
PPQGSQTHQVSLSKQPTSQSRGDPTGPKETSGHHHHHH.

⇒ *Nef - Tat - HIS*

DNA sequence (Seq. ID. No. 12)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGG
GGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAG
CCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCT
TGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACA
AAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCT
CAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGA
GGGGACCCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence(Seq. ID. No. 13)

MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWI
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLH
GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTA
CTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSR
GDPTGPKETSGHHHHHH.

E.coli-expressed constructs (fusion constructs)

⇒ *LipoD-Nef-HIS*

DNA sequence (Seq. ID. No. 14)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.
The Lipidation Signal Sequence is underlined. After processing, the cysteine
coded by the TGT codon, indicated with a star, becomes the amino terminal
residue which is then modified by covalently bound fatty acids.

```
                                                            *
ATGGATCCAAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGTTGT
AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT
GCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCTAAAGCACTT
GCTTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT
CGTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAA
TTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA
GAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGCAAGTGGTCA
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG
GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
GCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT
CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT
GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
GGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAG
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA
GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCACCATCACCAT
TAA
```

Protein sequence of the processed lipidated ProtD-Nef-HIS protein (Seq. ID. No. 15)

(Amino-acids corresponding to Prot D fusion partner are in bold)

```
CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE
EEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDP
EREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHHHHHH.
```

⇒ _LipoD-Nef-Tat-HIS_

DNA sequence (Seq. ID. No. 16)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.
The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

                                                                                          ✷

<u>ATGGATCCAAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGTTGT</u>
AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT
GCTCACCGTGGTGCTAGCGGTTATTACCAGAGCATACGTTAGAATCTAAAGCACTT
GCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT
CGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAA
TTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA
GAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGCAAGTGGTCA
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG
GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
GCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT
CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT
GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
GGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAG
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA
·GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTAGACTA
GAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTGT
AAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCC
TATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCAT
CAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCG
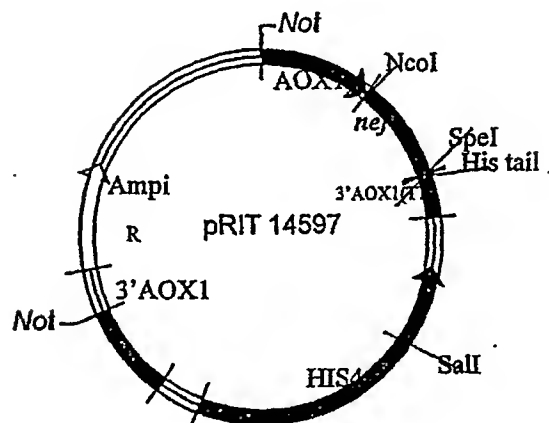AAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein <small>sequence</small> of the processed lipidated ProtD-NEF-TAT-HIS protein (Seq. ID. No. 17)

(Amino-acids corresponding to Prot D fusion partner are in bold)

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE
EEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDP
EREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCY
CKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTG
PKETSGHHHHHH.

⇒ *ProtD-Nef -HIS*

DNA sequence (Seq. ID. No. 18)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA
ATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCT
AAAGCACTTGCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGGAATGACT
AAGGATGGTCGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTT
GCGAAAAAATTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT
ACCTTAAAAGAAATTCAAAGTTTAGAAATGACAGAAACTTTGAAACCATGGGTGGC
AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA
GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA
GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA
CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG
ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAA
GGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA
CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCA
CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG
GCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT
GACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCAC
GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCAC
CATCACCATTAA

Protein sequence (Seq. ID. No. 19)

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL
EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLK
EIQSLEMTENFETMGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDL
EKHGAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSH
FLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGW
CYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFDSRLAFH
HVARELHPEYFKNCTSGHHHHHH.

⇒ *ProtD-Nef -Tat-HIS*

DNA sequence (Seq. ID. No. 20)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

```
ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA
ATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCT
AAAGCACTTGCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACT
AAGGATGGTCGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTT
GCGAAAAAATTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT
ACCTTAAAAGAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGC
AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA
GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA
GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA
CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG
ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAA
GGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA
CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCA
CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG
GCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT
GACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCAC
GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGAT
CCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCAAT
TGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTA
GGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGT
CAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCG
ACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA
```

Protein sequence (Seq. ID. No. 21)

(Amino-acids corresponding to Prot D fusion partner are in bold)

```
MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMT
KDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGG
KWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEA
QEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHT
QGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMD
DPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTN
CYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDP
TGPKETSGHHHHHH.
```

⇒ *Tat-MUTANT-HIS*

DNA sequence (Seq. ID. No. 22)

```
ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATC    40
CAGGAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTG    80
TAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACA   120
GCTGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGAC   160
AGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGT   200
TTCTCTATCAAAGCAACCCACCTCCCAATCCAAAGGGGAG   240
CCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATC   280
ACCATTAA                                   288
```

Protein sequence(Seq. ID. No. 23)

Mutated amino-acids in Tat sequences are in bold.

```
MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFIT    40
AALGISYGRKKRQRRRPPQGSQTHQVSLSKQPTSQSKGE    80
PTGPKETSGHHHHHH.                            95
```

⇒Nef-Tat-Mutant-HIS

DNA sequence(Seq. ID. No. 24)

```
ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGC    40
CTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGC    80
AGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACAT   120
GGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTG   160
CTTGTGCCTGGCTAGAAGCACAAGAGGAGGAGGAGGTGGG   200
TTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACT   240
TACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAA   280
AGGGGGGACTGGAAGGGCTAATTCACTCCCAACGAAGACA   320
AGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC   360
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCA   400
GATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACC   440
AGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAG   480
AACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGG   520
ATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAG   560
CCGCCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCG   600
GAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTA   640
GACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAAC   680
TGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT   720
TGCCAAGTTTGTTTCATAACAGCTGCCTTAGGCATCTCCT   760
ATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCA   800
AGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCC   840
ACCTCCCAATCCAAAGGGGAGCCGACAGGCCCGAAGGAAA   880
CTAGTGGCCACCATCACCATCACCATTAA              909
```

Protein sequence (Seq. ID. No. 25)
Mutated amino-acids in Tat sequence are in bold.

```
MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKH   40
GAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMT   80
YKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGY  120
FPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGE  160
NTSLLHPVSLHGMDDPEREVLEWRFDSRLAFHHVARELHP  200
EYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFH  240
CQVCFITAALGISYGRKKRRQRRRPPQGSQTHQVSLSKQP  280
TSQSKGEPTGPKETSGHHHHHH.                   302
```

Figure 2

Map of pRIT14597 integrative vector

MCS POLYLINKER: *nef* gene inserted between NcoI and SpeI sites.

```
AsuII      NcoI            SpeI                                          EcoRI
TTCGAA.ACC.ATGGCCGCGGACTAGTGGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGGAATTC
           Thr .Ser . Gly.  His .  His . His . His  . His . His
```

The amino acid sequence of Figure 3 relates to Seq. ID no. 27 and the nucleic acid sequence of Figure 3 relates to Seq. ID. No. 26.

Figure 3 : SDS-PAGE: Nef-Tat-his fusion protein
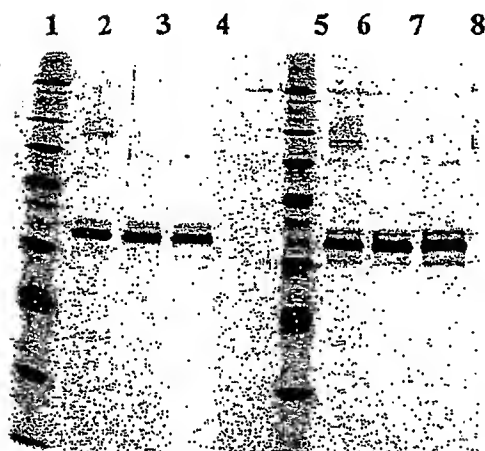
1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa
2: TNH/23 SP eluate (250 ng)
3: TNH/23 Purified bulk (250 ng)
4: TNH/22 Purified bulk (250 ng)

5: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa
6: TNH/23 SP eluate (400 ng)
7: TNH/23 Purified bulk (400 ng)
8: TNH/22 Purified bulk (400 ng)

Daiichi Silver Staining
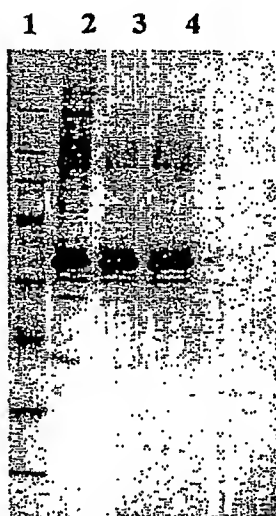
Blot α Nef-Tat (LAS 97340)
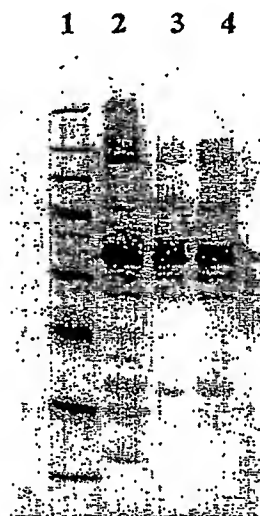
Blot Tat2

Figure 4 : SDS-PAGE: Nef-Tat-his fusion protein
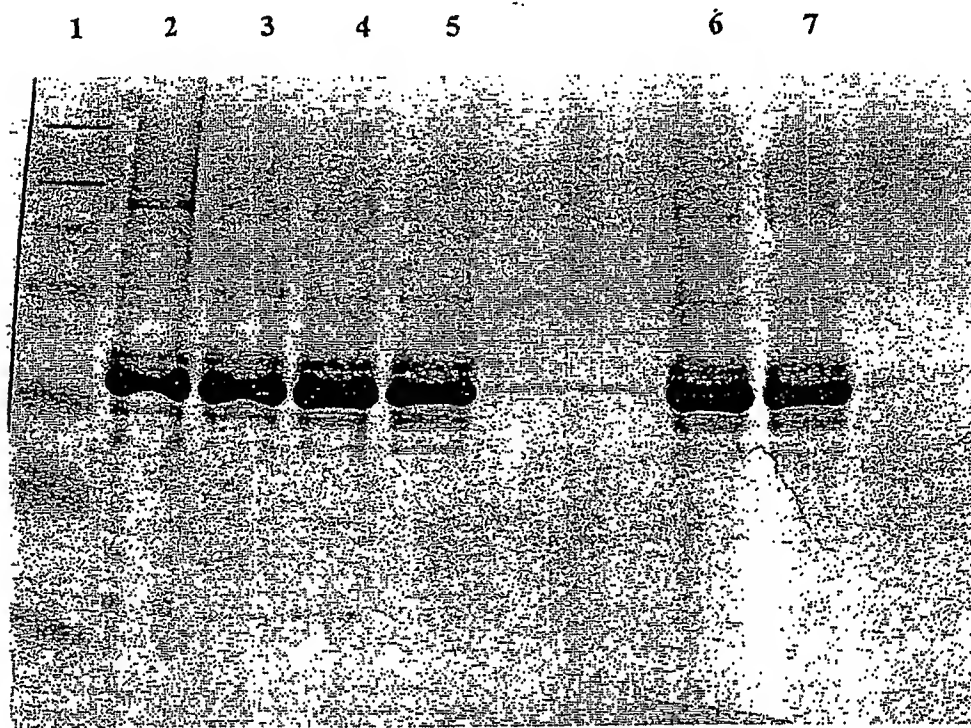
Coomassie blue G250

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: TNH/23 SP eluate (4 µg)
3: TNH/23 Superdex200 eluate (4 µg)
4: TNH/23 Purified bulk (4 µg)
5: TNH/22 Purified bulk (4 µg)

6: TNH/23 Purified bulk (4 µg) / non reducing conditions
7: TNH/22 Purified bulk (4 µg) / non reducing conditions

Figure 6:   SDS-PAGE ANALYSIS – reducing conditions
(14% polyacrylamide precasted gels - Novex) See example 5
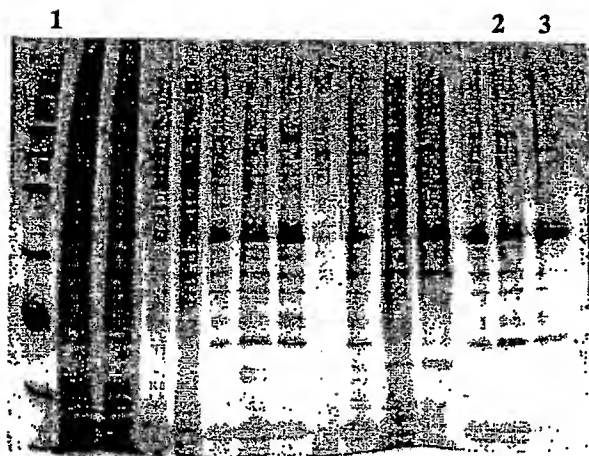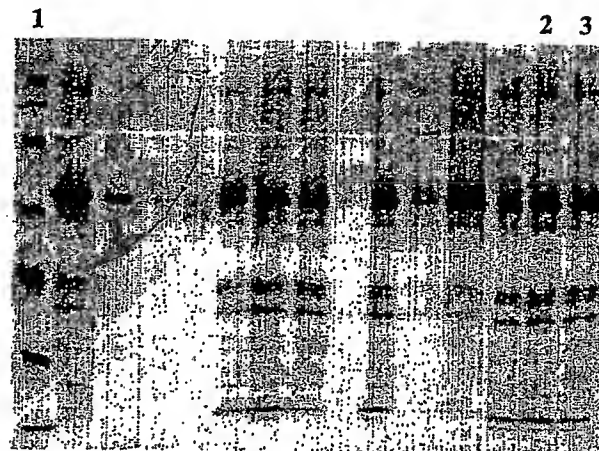
Silver staining   Western blot α Tat

1: MW (175/83/62/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk
3: Purified bulk

Figure 7 (relating to Example 6): SDS-PAGE ANALYSIS:
(4-20% polyacrylamide precasted gels - Novex)

Coomassie blue G250

Western blot anti Tat
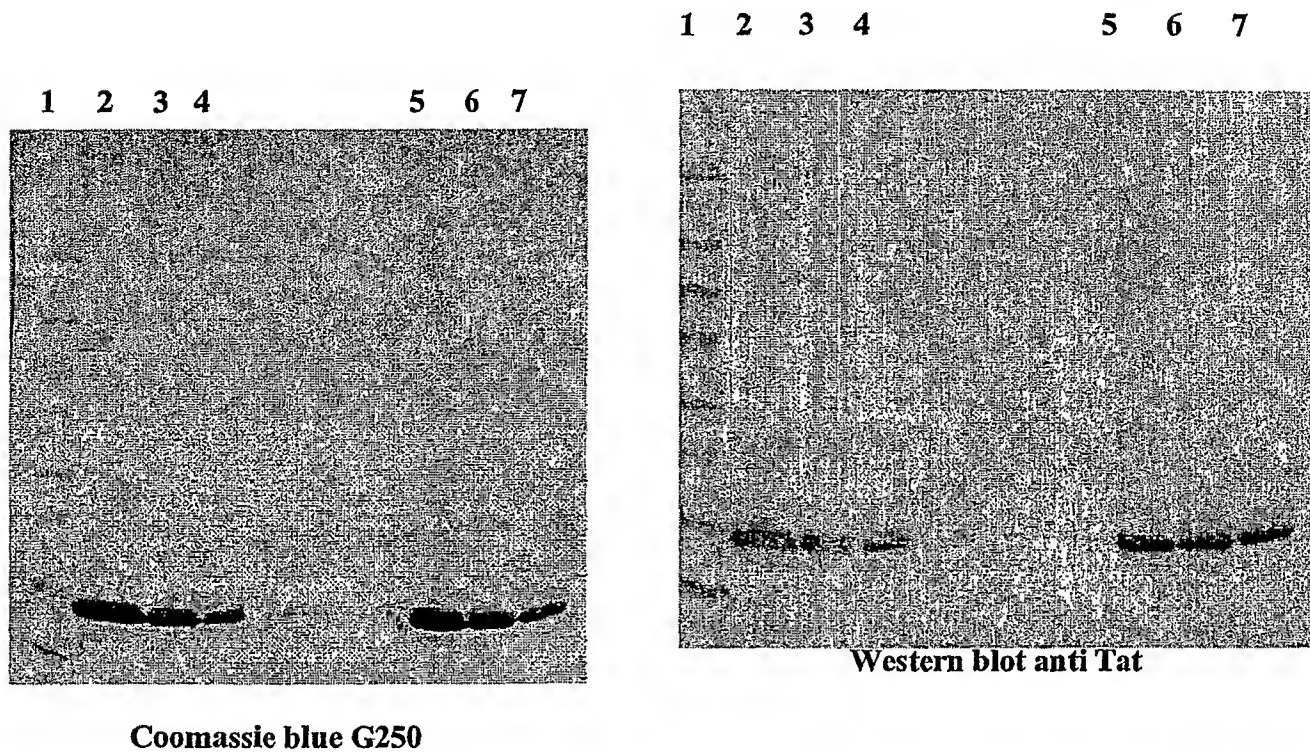
1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk (reducing conditions)
3: Purified bulk (reducing conditions)
4: Purified bulk (reducing conditions)

5: Purified bulk (non reducing conditions)
6: Purified bulk (non reducing conditions)
7: Purified bulk (non reducing conditions)

Figure 8 (relating to Example 7): SDS-PAGE ANALYSIS:
(4-20% polyacrylamide precasted gels - Novex)
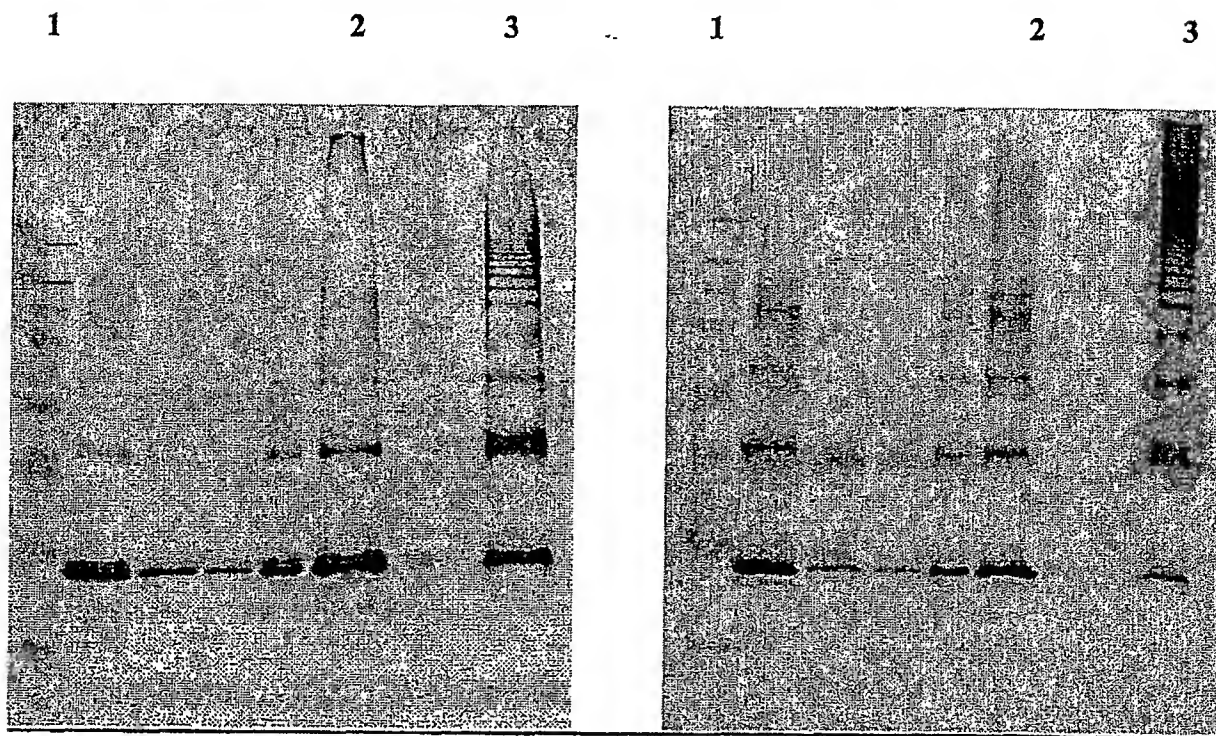
Coomassie blue G250   **Western blot anti Tat**

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk (reducing conditions)
3: Purified bulk (non reducing conditions)

FIGURE 9: SDS-PAGE ANALYSIS - REDUCING CONDITIONS

(14% polyacrylamide precasted gels - Novex) see Example 8
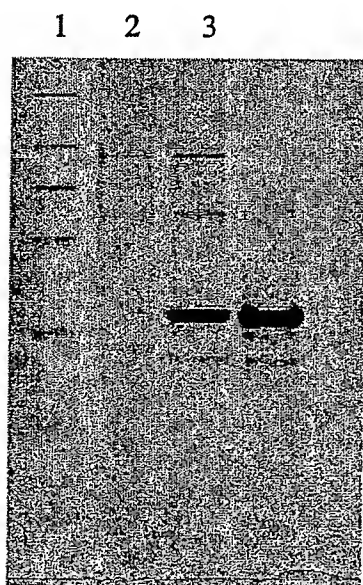
Coomassie blue R250
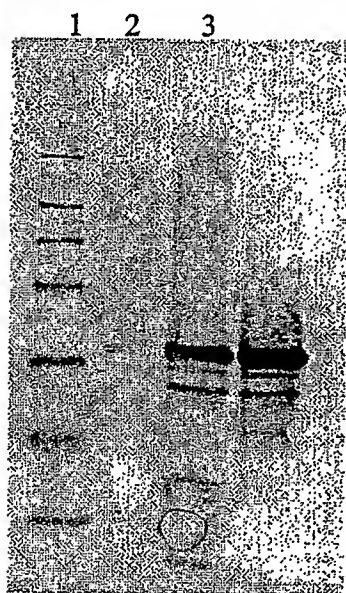
Silver staining

Figure 10: SDS-PAGE ANALYSIS – reducing conditions

(14% polyacrylamide precasted gels - Novex) See Example 9

Coomassie blue G250   **Silver staining**   **W. Blot α Nef**
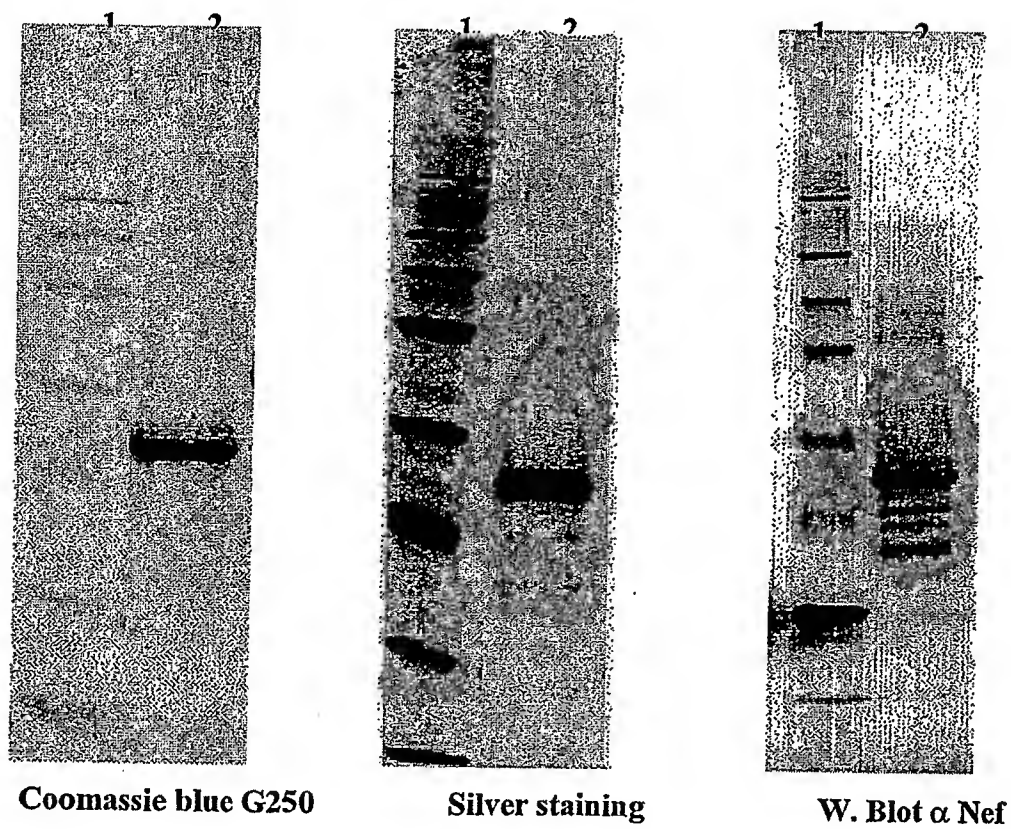
1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk

Figure 11

Map of pRIT14908 integrative vector
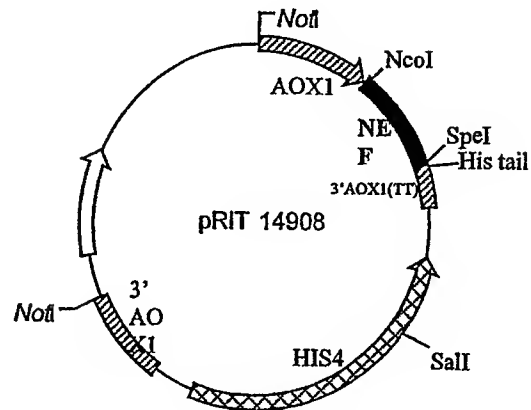
MCS POLYLINKER : NEF gene inserted between NcoI and SpeI sites.

| Asu II | Nco I | | Spe I | | | Eco RI |

TTCGAA.A CC.ATGGCCGCGG ACTAGT .GGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGC GAATTC

Thr .Ser . Gly. His . His . His . His ..His . His .

Figure 12

Sequences of Pichia-expressed SIV-NEF-His protein

DNA SEQUENCE:

```
atgggtggagctatttccatgaggcggtccaggccgtctggagatctgcg      50
acagagactcttgcgggcgcgtggggagacttatgggagactcttaggag     100
aggtggaagatggatactcgcaatccccaggaggattagacaagggcttg     150
agctcactctcttgtgagggacagaaatacaatcagggacagtatatgaa     200
tactccatggagaaacccagctgaagagagagaaaaattagcatacagaa     250
aacaaaatatggatgatatagatgaggaagatgatgacttggtaggggta     300
tcagtgaggccaaaagttcccctaagaacaatgagttacaaattggcaat     350
agacatgtctcattttataaaagaaaaggggggactggaagggatttatt     400
acagtgcaagaagacatagaatcttagacatatacttagaaaaggaagaa     450
ggcatcataccagattggcaggattacacctcaggaccaggaattagata     500
cccaaagacatttggctggctatggaaattagtccctgtaaatgtatcag     550
atgaggcacaggaggatgaggagcattatttaatgcatccagctcaaact     600
tcccagtgggatgacccttggggagaggttctagcatggaagtttgatcc     650
aactctggcctacacttatgaggcatatgttagatacccagaagagtttg     700
gaagcaagtcaggcctgtcagaggaagaggttagaagaaggctaaccgca     750
agaggccttcttaacatggctgacaagaaggaaactcgcactagtggcca     800
ccatcaccatcaccattaa.                                   819
```

PROTEIN SEQUENCE:

```
MGGAISMRRSRPSGDLRQRLLRARGETYGRLLGEVEDGYSQSPGGLDKGL      50
SSLSCEGQKYNQGQYMNTPWRNPAEEREKLAYRKQNMDDIDEEDDDLVGV     100
SVRPKVPLRTMSYKLAIDMSHFIKEKGGLEGIYYSARRHRILDIYLEKEE     150
GIIPDWQDYTSGPGIRYPKTFGWLWKLVPVNVSDEAQEDEEHYLMHPAQT     200
SQWDDPWGEVLAWKFDPTLAYTYEAYVRYPEEFGSKSGLSEEEVRRRLTA     250
RGLLNMADKKETRTSGHHHHHH.                                272
```

Figure 13
Coomassie Blue Stained SDS-PAGE of recombinant Pichia pastoris SIV/NEF expressing strains
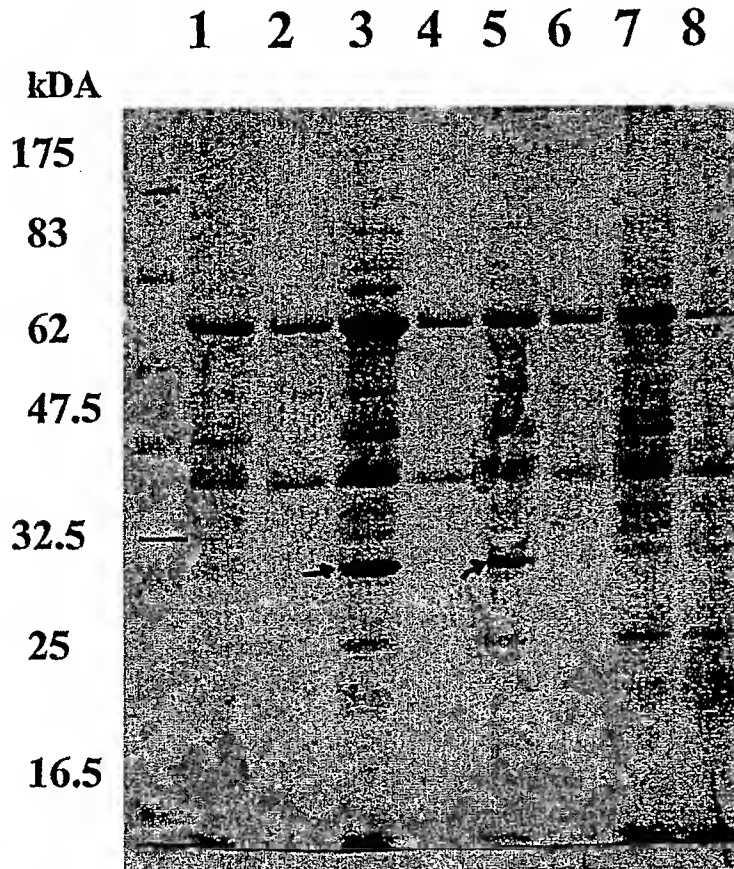
lane 1: P-   Y1752 strain
lane 2: S-   "   "
lane 3: P-   Y1772 strain
lane 4: S-   "   "   "

lane 7: P-   GS115 strain (negative control)
lane 8: S-   "   "

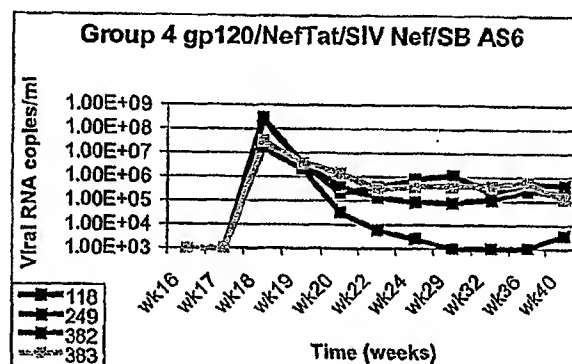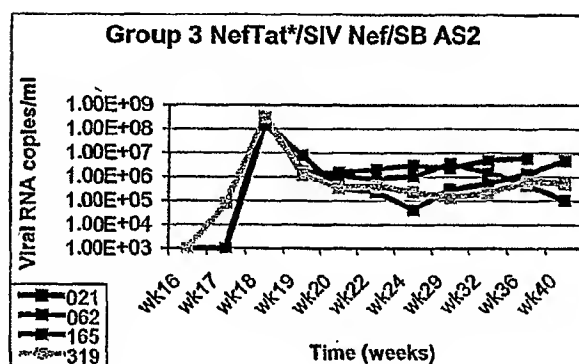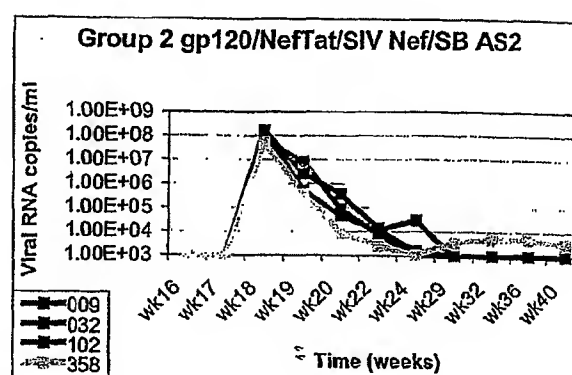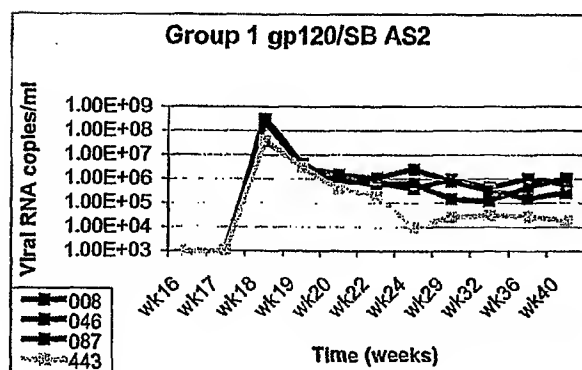Figure 14. Monkey study 1. Analysis of CD4-positive cells among PBMCs before and after challenge with SHIV
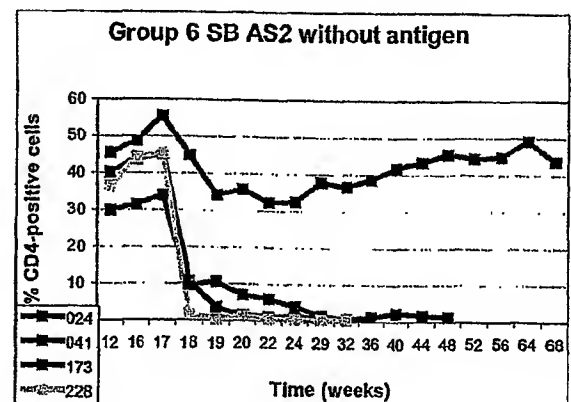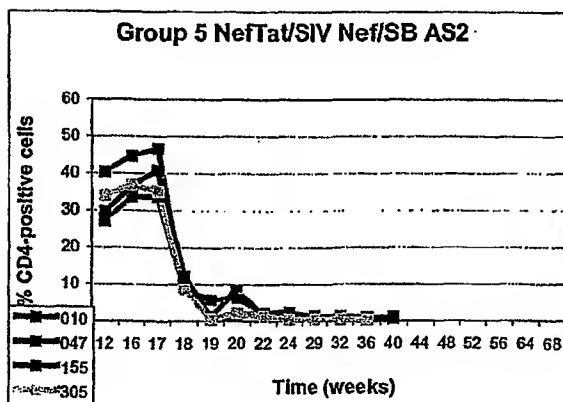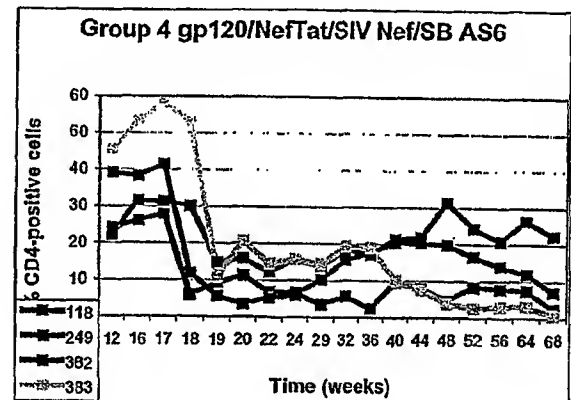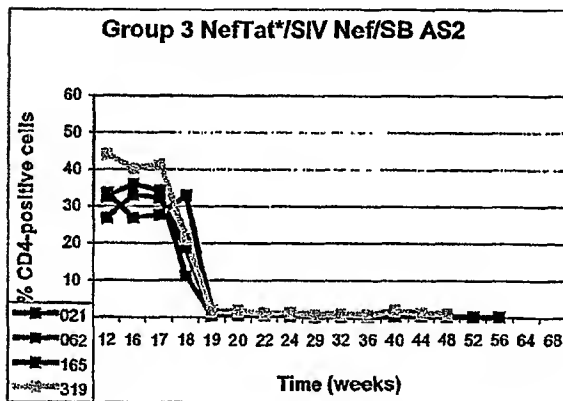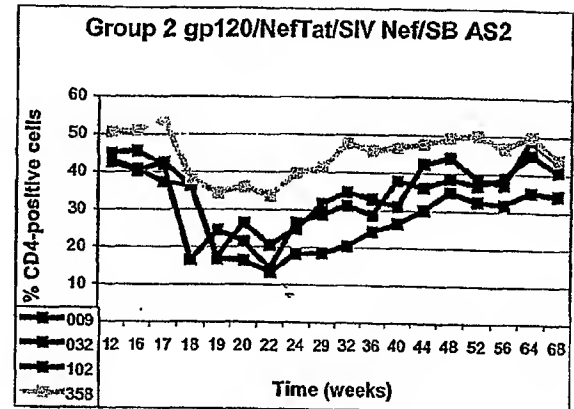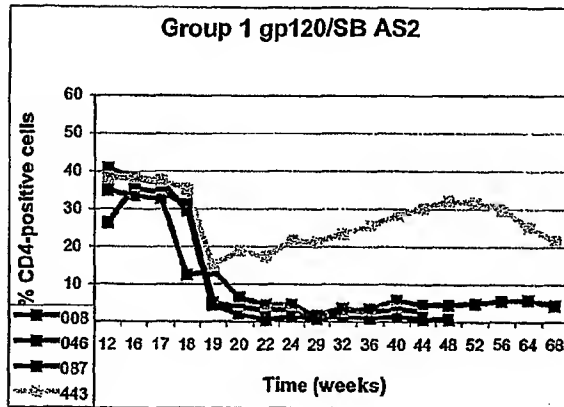
Figure 15. Monkey study 1. Analysis of SHIV plasma virus load after challenge with SHIV

Group 1 gp120/SB AS2

Group 2 gp120/NefTat/SIV Nef/SB AS2

Group 3 NefTat*/SIV Nef/SB AS2

Group 4 gp120/NefTat/SIV Nef/SB AS6

Group 5 NefTat/SIV Nef/SB AS2

Group 6 SB AS2 without antigen
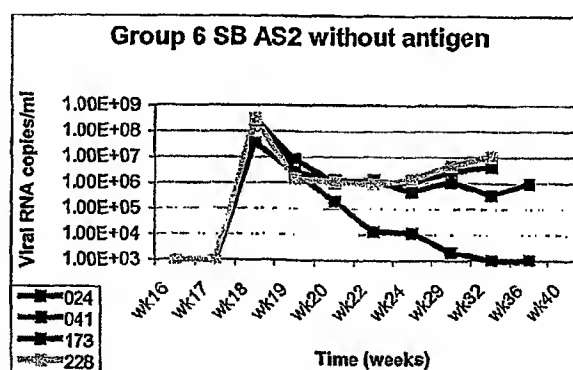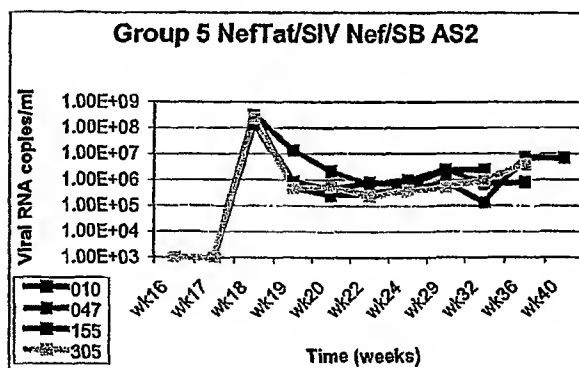
Figure 16. Monkey study 2. Analysis of CD4-positive cells among PBMCs before and after challenge with SHIV
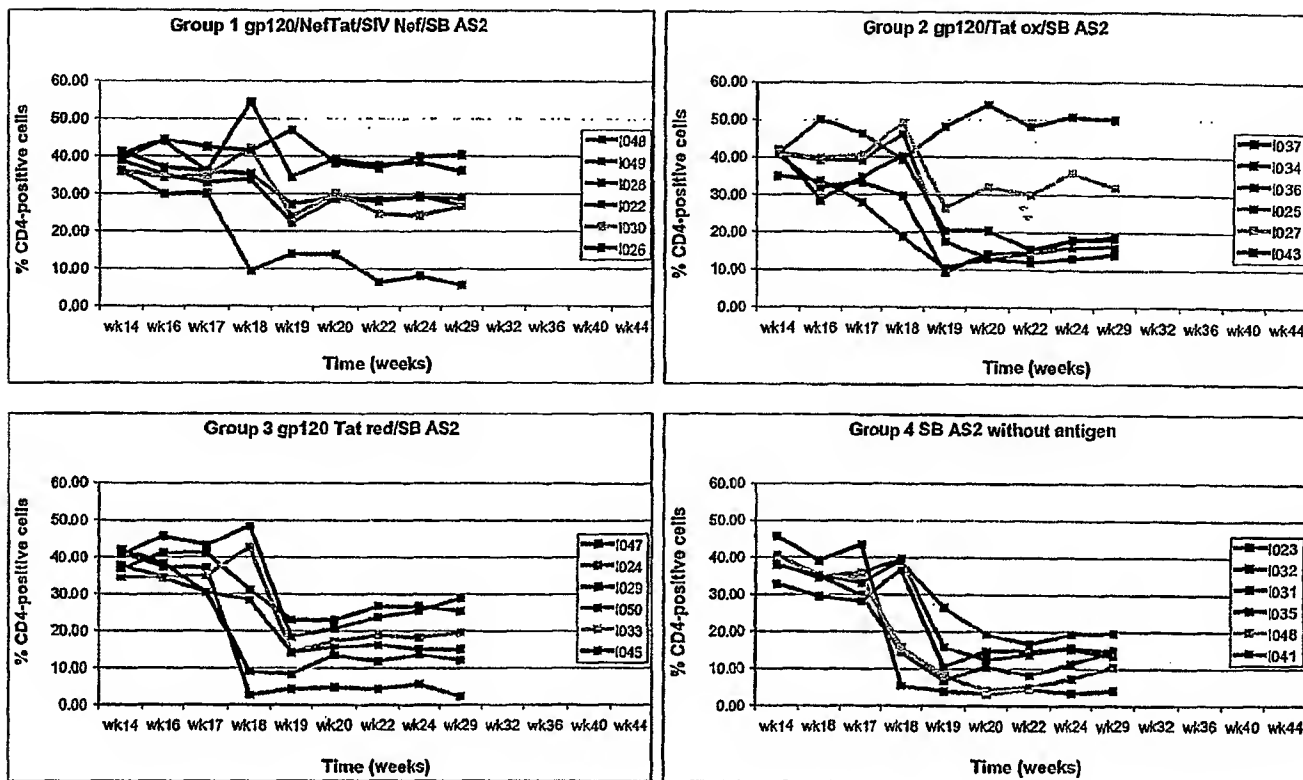
Figure 17. Monkey study 2. Analysis of SHIV plasma virus load after challenge with SHIV
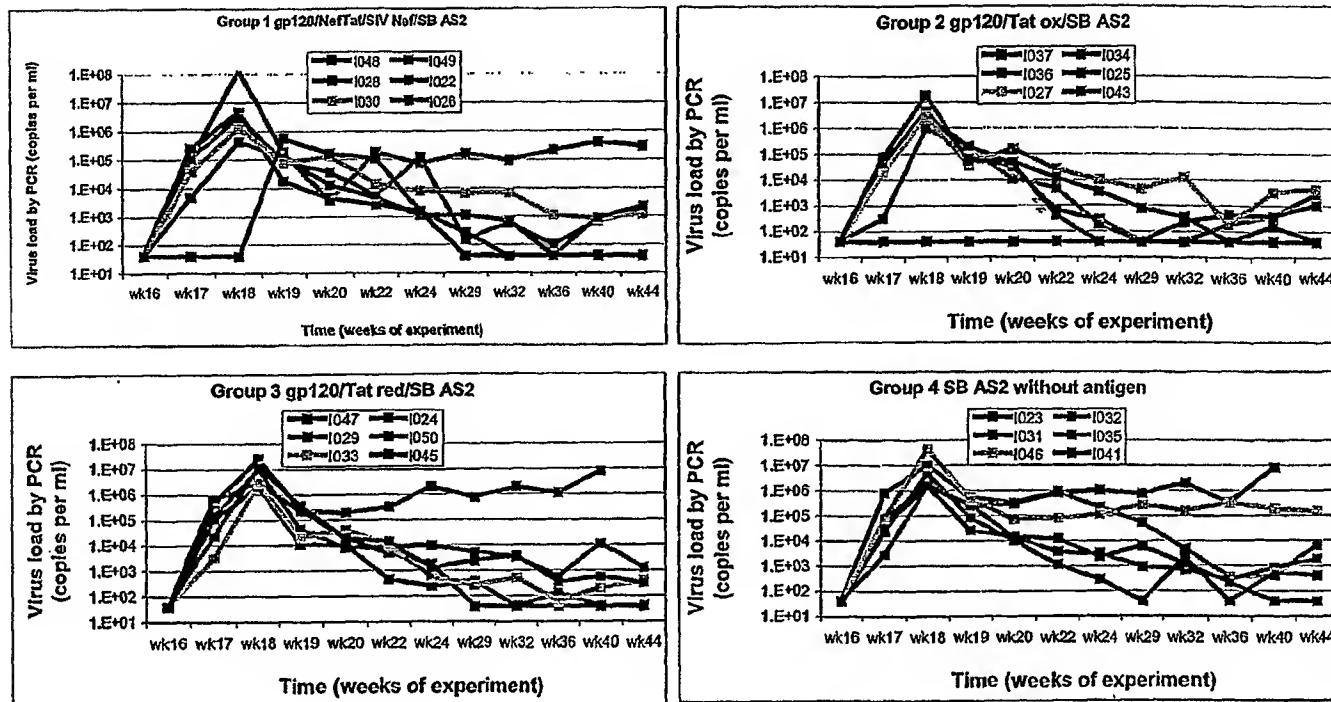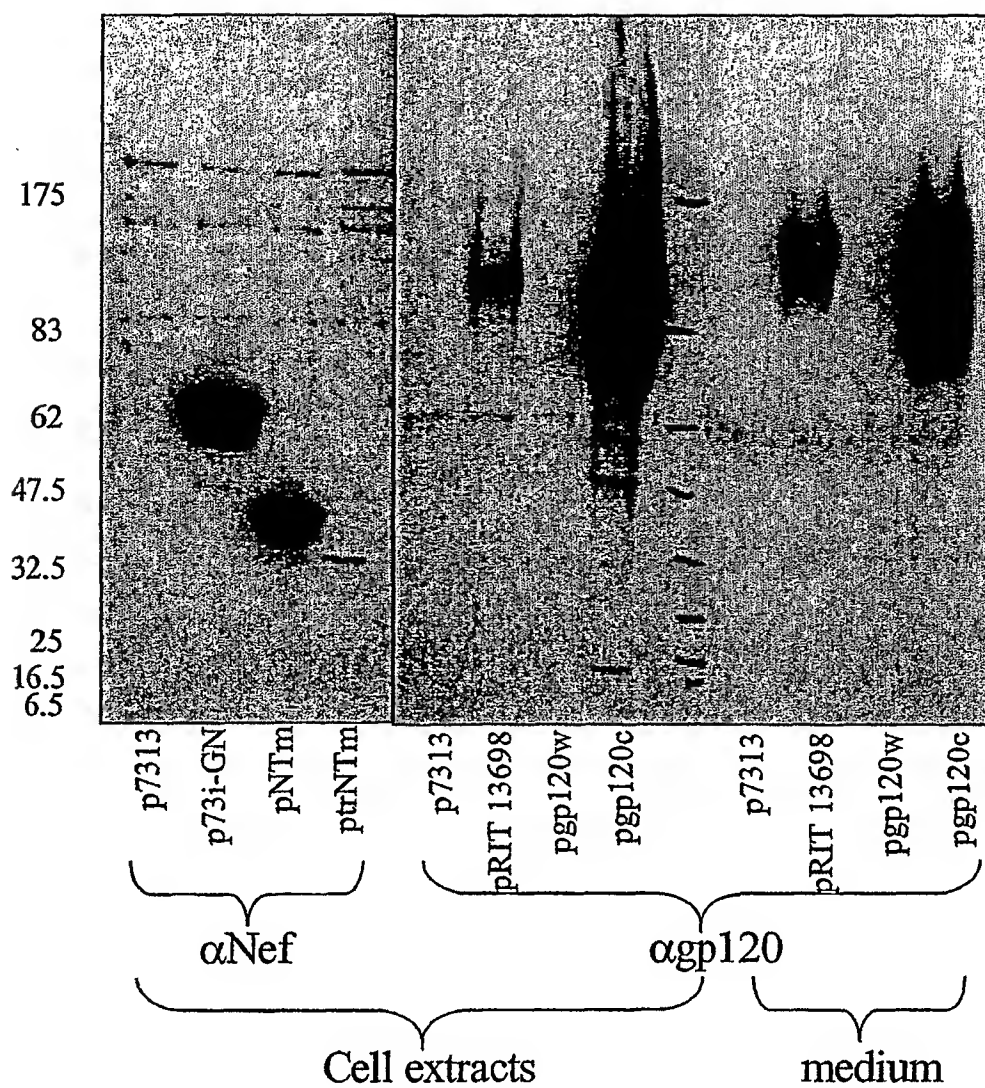
Fig. 18. Western blot showing the level of Nef detected in 293T cell extracts, and gp120 in cell extracts and supernatant medium, 24hr post transfection.
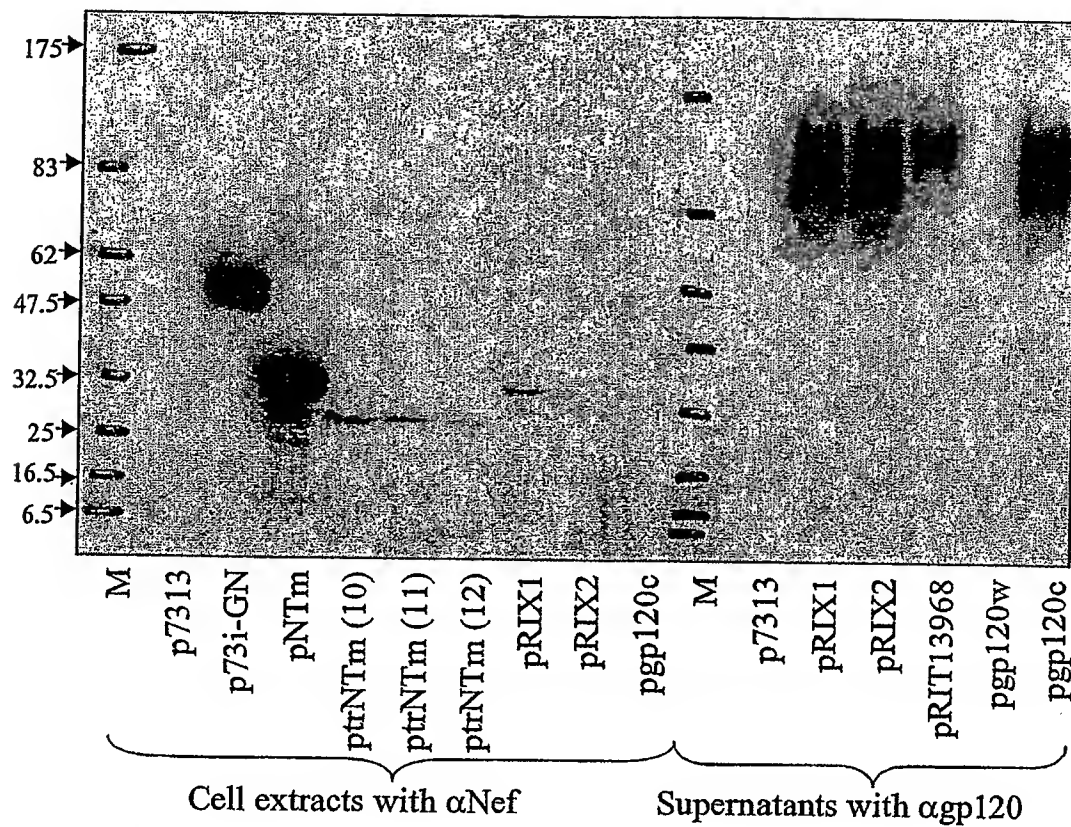
Fig. 19. Western blot showing the level of Nef detected in 293T cell extracts, and gp120 in supernatants, 24hr post transfection.
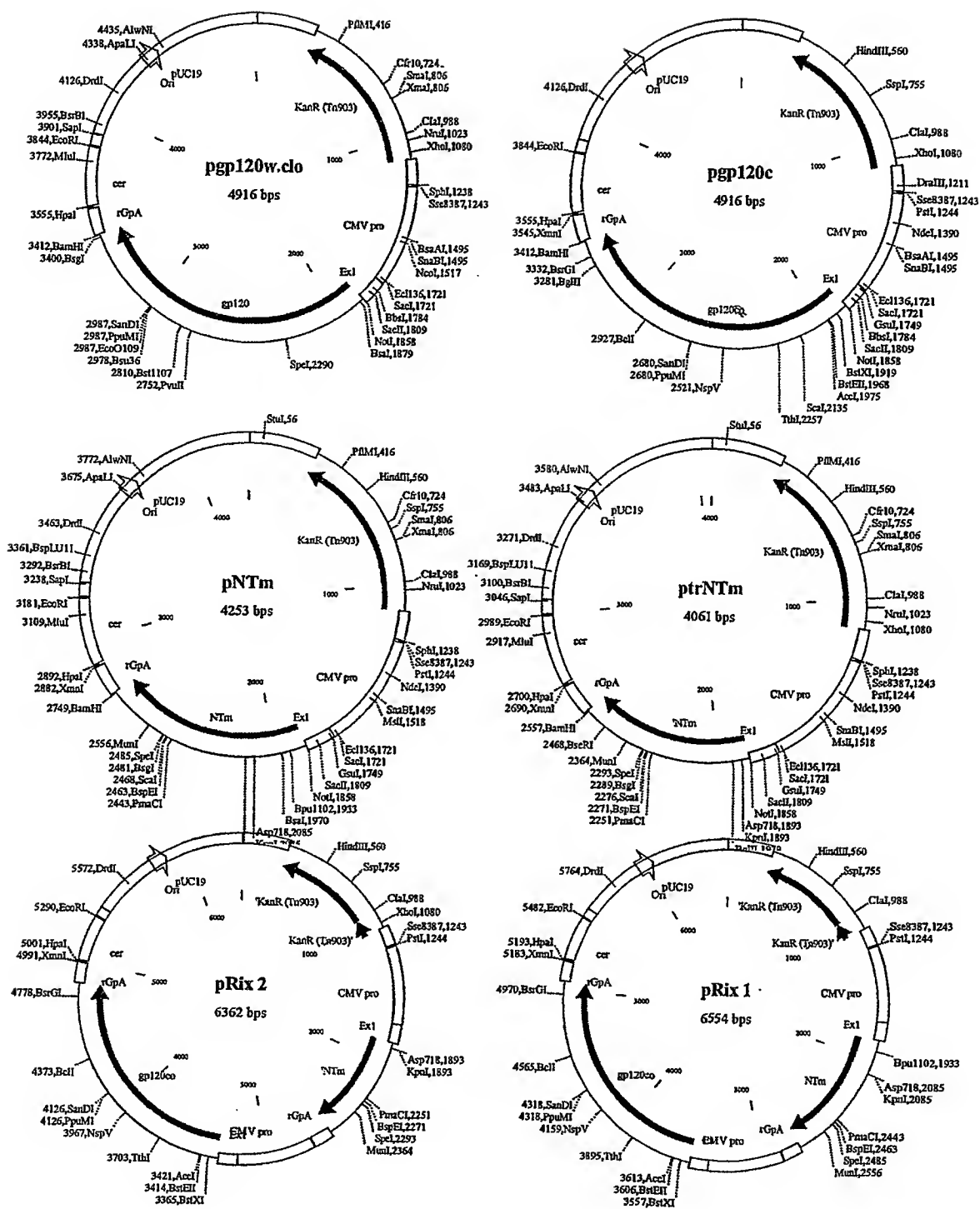
Fig.20. Plasmid Maps:

Fig. 21. Oligos for codon optimisation and sequencing of gp120:

```
g120-u:1    GAATTCGCGGCCGCAATGAAGGTCAAGGAGACCAGAAAGAACTACCAGCATCTGTG
g120-u:2    CCTGGGAATGCTGATGATCTGCTCCGCCGCCGAGCAGCTGTGGGTCACCGTCTACTACGG
g120-u:3    GGCCACGACCACCCTCTTCTGCGCGAGCGACGCCAAGGCCTACGACACGGAAGTGCATAA
g120-u:4    TTGCGTGCCTACGGGACCCCAACCCCCAGGAGGTGGTGCTGGGAAACGTGACCGAGTACTT
g120-u:5    CATGGTGGATCAGATGCACGAGGACATCATCTCTCTGTGGGACCAGTCCCTGAAGCCCTG
g120-u:6    CTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACCAGCAA
g120-u:7    TCGGAAGGGCGAGATCAAGAACTGCTCCTTCAATATCACGACCTCGATCAGAGACAAGGT
g120-u:8    GTTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATGCCACCACCAAGAACAAGAC
g120-u:9    CATTCACTGCAACAGCAGCGTCATGACGCAGGCCTGCCCCAAGGTGTCCTTCGAACCAAT
g120-u:10   CCCTGCCGGATTCGCGATCCTCAAGTGTAACAACAAGACCTTCGACGGGAAGGGCCTGTG
g120-u:11   GCAGTGCACCCATGGCATCCGCCCGTCGTGAGCACCCAGCTGCTGCTGAACGGGTCCCT
g120-u:12   GATCCGGTCGGACAACTTCATGGACAACACCAAGACAATCATCGTCCAGCTGAACGAGTC
g120-u:13   CCGGCCTAACAACAACACCCGTAAGGGCATCCACATCGGGGCCTGGACGGGCCTTCTATGC
g120-u:14   CGACATCCGGCAGGCCCATTGCAACCTCTCCCGCGCCCAGTGGAATAACACCCTGAAGCA
g120-u:15   AGAGCACTTTGGAAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCCGAGAT
g120-u:16   CTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGAACGG
g120-u:17   AGAGGGAAACTCCACTATCACCCTCCCTTGCCGCATCAAGCAGATCATCAACATGTGGCA
g120-u:18   GTATGCCCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCCAACATCACCGGCCTGCTGCT
g120-u:19   CGAGGGCAACGGCACGGAGAACGAGACGGAGATCTTCAGGCCCGGCGGCGGCGACATGAG
g120-u:20   GCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGGCGTGGCCCCCACCCGGGCCAA
g120-1:1    GAATTCGGATCCTCATCTCTGCACGACGCGGCGCTTGGCCCGGGTGGGGGCCACG
g120-1:2    ACCACCTTGTACTTGTACAGCTCGCTCCGCCAGTTATCCCTCATGTCGCCGCCGCCGGGC
g120-1:3    TTCTCCGTGCCGTTGCCCTCGGTGCCCCCGTCTCTGGTGAGCAGCAGGCCGGTGATGTTG
g120-1:4    CCCCCGATGGGGGGGGGCATACATGGCCTTTCCCACCTCCTGCCACATGTTGATGATCTGC
g120-1:5    GTGATAGTGGAGTTTCCCTCTGTGTTGTTGCCCTCGGTGCCGTTCCAGGTGGAGTTGAAG
g120-1:6    TAGAAGAACTCGCCCCCGCAGTTGAAGGAGTGCCGCACGATCTCGGGGTCTCCGCCAGAA
g120-1:7    GTCTTGTTTCCAAAGTGCTCTCTCAGCTTGATCACGATCTGCTTCAGGGTGTTATTCCAC
g120-1:8    CAATGGGCCTGCCGGATGTCGCCGATGATCTTGCGGGCGGCATAGAAGGCCCGTCCAGGC
g120-1:9    CGGGTGTTGTTGTTAGGCCGGGTACAGTTAATCGCCACAGACTCGTTCAGCTGGACGATG
g120-1:10   ATGAAGTTGTCCGACCGGATCACCCACCTCCTCCTCAGCCAGGGACCCGTTCAGCAGCAGC
g120-1:11   CGGATGCCATGGGTGCACTGCACCGTGCTGACGTTGGTGCACAGGCCCTTCCCGTCGAAG
g120-1:12   AGGATCGCGAATCCGGCAGGGGCACAGTAATGGATCGGGATTGGTTCGAAGGACACCTTG
g120-1:13   ACGCTGCTGTTGCAGTGAATGAGTCTGAAATTACGCGTCGTCTTGTTCTTGGTGGTGGCA
g120-1:14   ACCACATCGAGATTATAAAACAGCGCGTATTCCTTCTGCACCTTGTCTCTGATCGAGGTC
g120-1:15   TTCTTGATCTCGCCCTTCCGAATCTCTCCGGTCCAGCCGTTGCTGGTGGTGGTAGTGCTG
g120-1:16   TCACAGTCCAGTGTCACGCAGAGAGGCGTCAGCTTCACGCAGGGCTTCAGGGACTGGTCC
g120-1:17   TCGTGCATCTGATCCACCATGTTATTCTTCCACATGTTGAAGTACTCGGTCACGTTTCCC
g120-1:18   TTGGGGTCCGTAGGCACGCAAGCATGCGTCGCCCACACGTTATGCACTTCCGTGTCGTAG
g120-1:19   CAGAAGAGGGTGGTCGTGGCCTCCTTCCACACAGGCACGCCGTAGTAGACGGTGACCCAC
g120-1:20   CAGATCATCAGCATTCCCAGGAGCATGGTGCCCCAGCGCCACAGATGCTGGTAGTTCTTT
```

Sequencing primers for optimised gp120

```
g120c-SF1   cggcgtgcctgtgtggaaggaggc
g120c-SF2   gacaaggtgcagaaggaatacgcg
g120c-SF3   cgtccagctgaacgagtctgtggc
g120c-SF4   catgtggcaggaggtgggaaaggcc
g120c-SR1   gtgagcagcaggccggtgatgttgg
g120c-SR2   cggcatagaaggcccgtccaggcc
g120c-SR3   catgaccgctgctgttgcagtgaatg
g120c-SR4   aggcacgcaagcatgcgtcgcccac
```

Sequencing primers for wild type gp120

```
g120w-SF1   ccactctattctgtgcatcagatgc
g120w-SF2   ccttgatgtagtaccaatagatgatg
g120w-SF3   ggtatacatataggaccagggagagc
g120w-SF4   agatgttcatcaaatattacagggc
g120w-SR1   catatctcctcctccaggtctgaag
g120w-SR2   gttattccattgtgctctactaag
g120w-SR3   ggacaggcctgtgtcatgactgag
g120w-SR4   ctacttcttgtgggttggggtctg
```
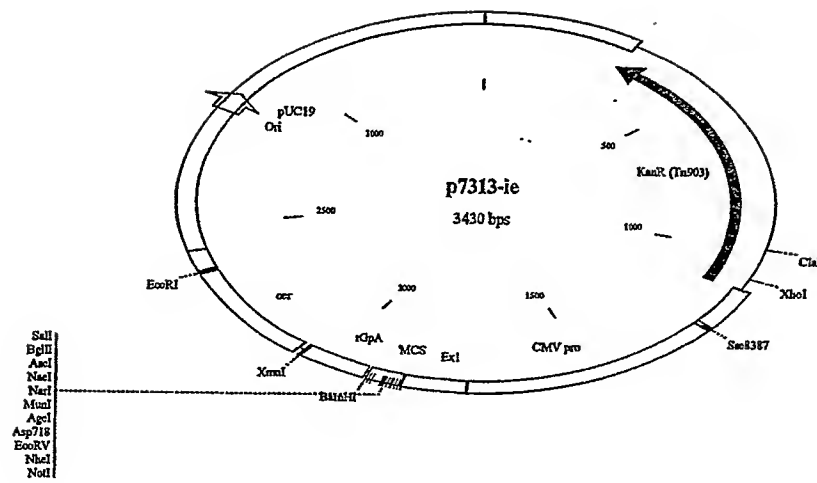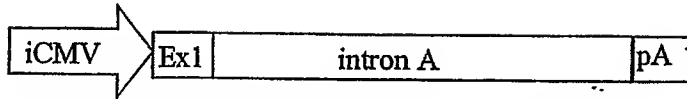
Figure 22 -- Map of p7313

Figure 23: Typical expression plasmid for antigens

Schematic representation of the standard expression cassette used for antigen expression. The gene of interest was inserted into a plasmid 3' to a enhanced HCMV IE1 promoter (iCMV + Exon 1) (nucleotides -489 to +118 relative to the transcription start), and 5' to a rabbit beta globin polyadenylation signal (pA). The plasmid backbone additionally contained a pUC19 origin of replication and a kanomycin selection marker.

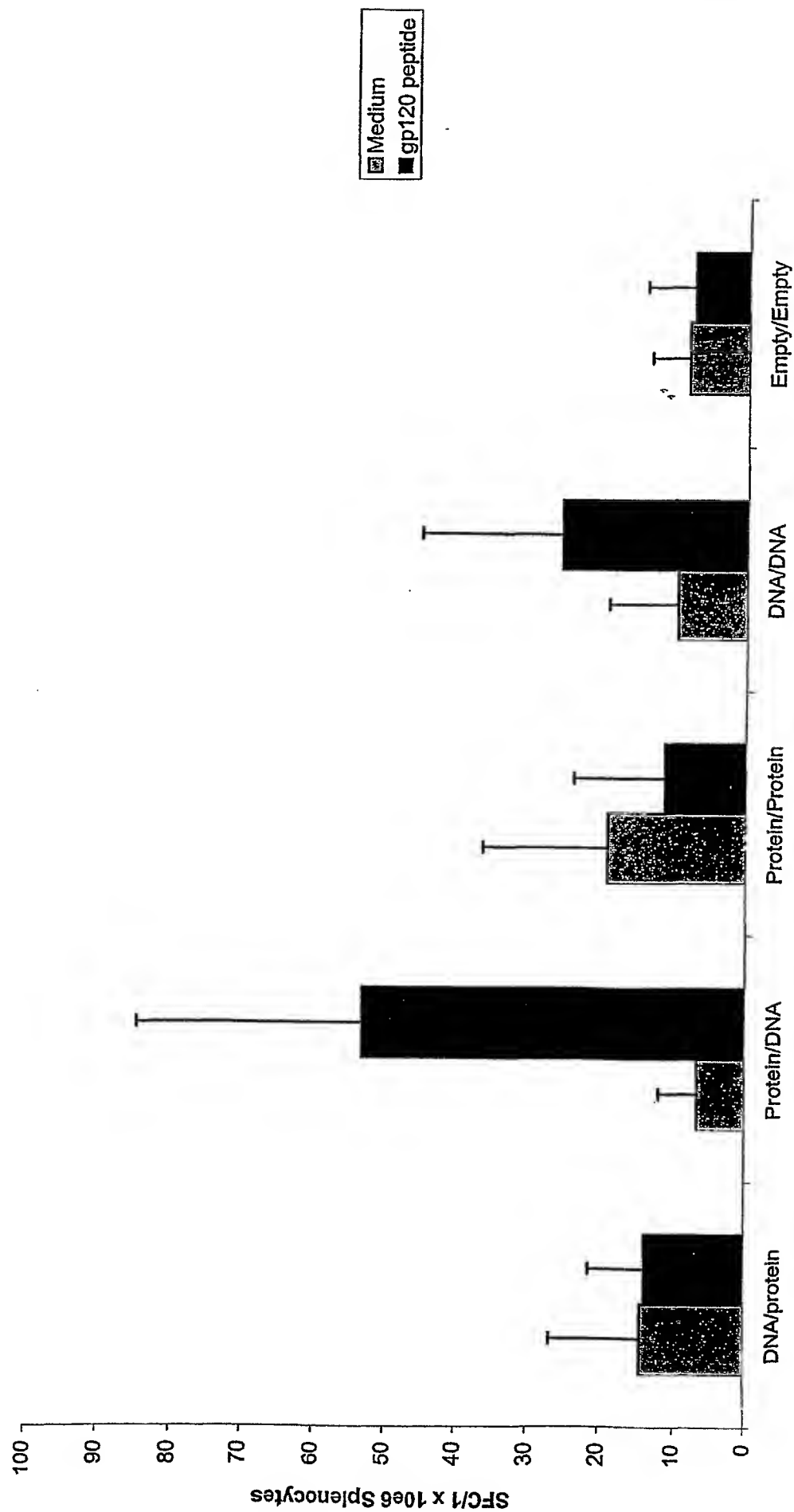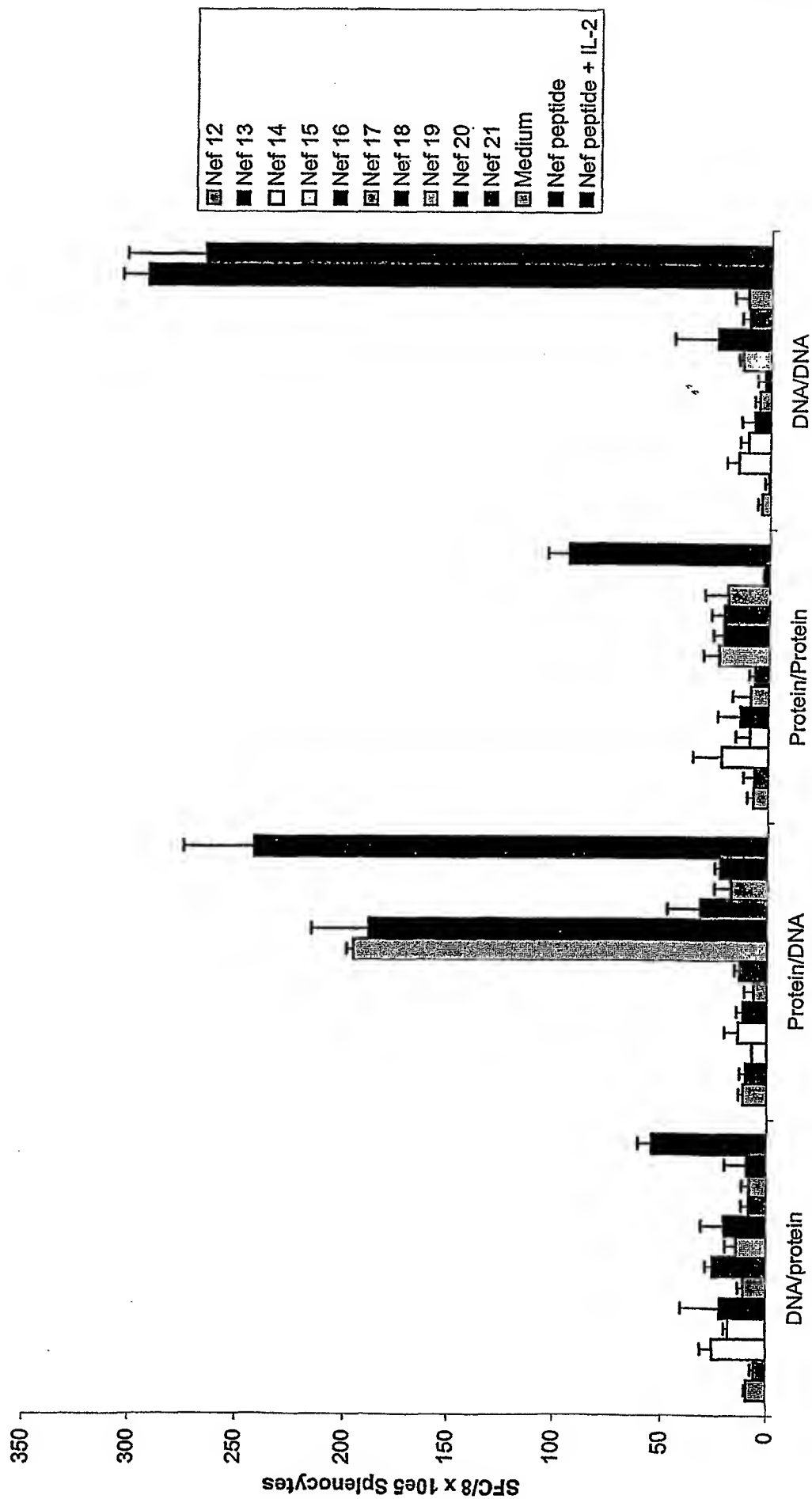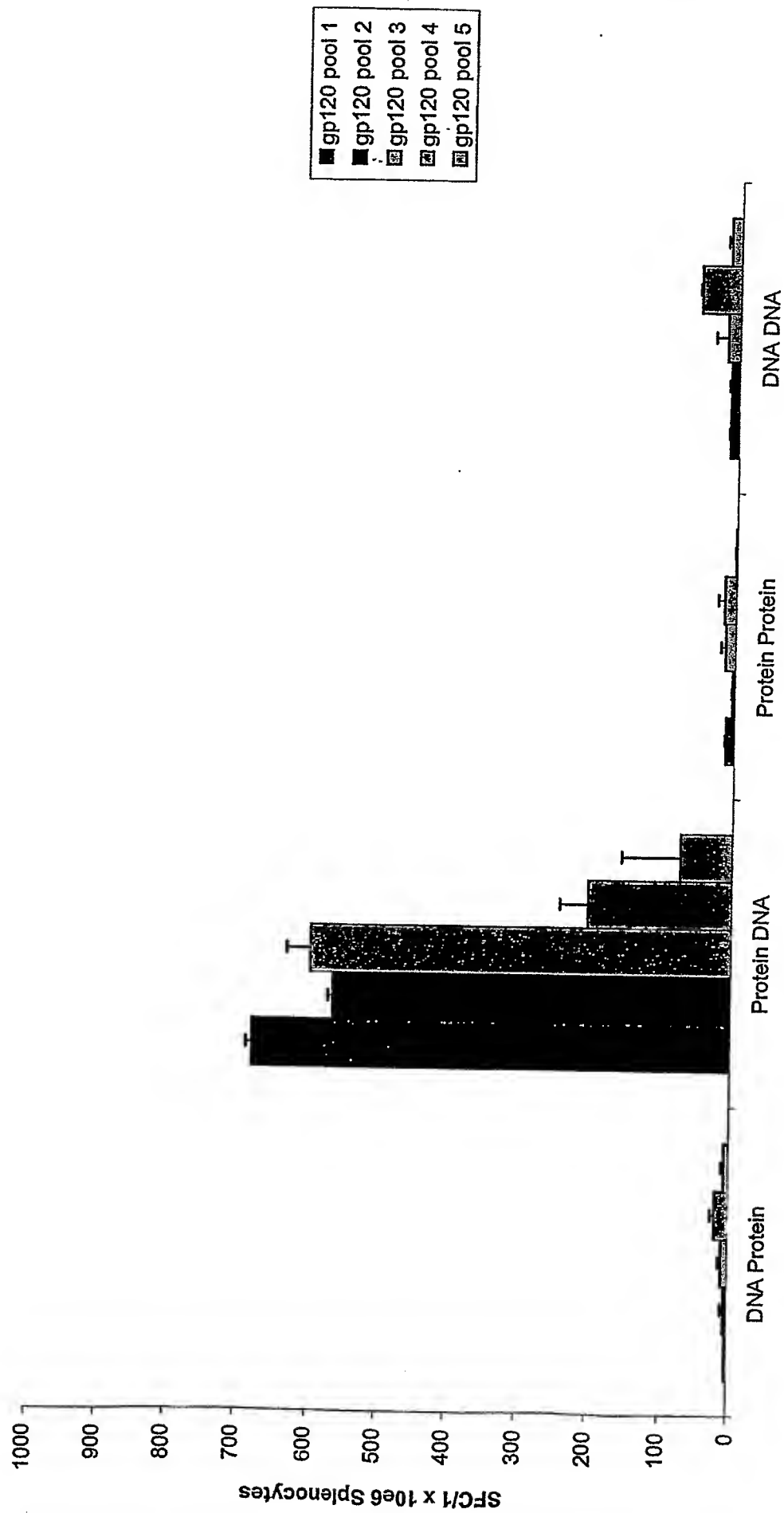Figure 24: Responses of immunised F1 (Balb/c x C3H) mice to gp120 peptide by IFN-gamma ELIspot at 5 days post-boost

Figure 25: Responses to 20-mer Nef peptide library by IFN-gamma ELIspot at 5 days post-boost

Figure 26: Responses of immunised Balb/c mice to gp120 peptide pools by IFN-gamma ELIspot at 7 days post-boost

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   A61K39/21   A61K31/713   C12N15/49   C12N15/89   C07K14/16
          C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   A61K   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

WPI Data, EPO-Internal, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 16884 A (GODART STEPHANE ANDRE GEORGES ;SMITHKLINE BEECHAM BIOLOG (BE); BRU) 8 April 1999 (1999-04-08) page 8, line 12,13.	1-11
X	HEYDENBURG FULLER D ET AL: "A QUALITATIVE PROGRESSION IN HIV TYPE 1 GLYCOPROTEIN 120-SPECIFIC CYTOTOXIC CELLULAR AND HUMORAL IMMUNE RESPONSES IN MICE RECEIVING ADNA-BASED GLYCOPROTEIN 120 VACCINE" AIDS RESEARCH AND HUMAN RETROVIRUSES, NEW YORK, NY, US, vol. 10, no. 11, 1 November 1994 (1994-11-01), pages 1433-1441, XP000572444 ISSN: 0889-2229	12-16
Y	the whole document	1-11, 17-22

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

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'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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Date of the actual completion of the International search	Date of mailing of the International search report
3 January 2003	16/01/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Bilang, J

International Application No

PCT/EP 02/08343

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